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OBSERVATIONS ON THE LEPTOMENINGEAL COLLATERAL CIRCULATION IN DOGS

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Anastomoses in the leptomeninges between branches of the anterior, middle and posterior cerebral arteries normally occur in man and other mammals. This potential collateral circulation to the cortex was described initially by Heubner (1872). Its functional significance has remained much in dispute, gaining acceptance only lately, largely from the work of Vander Eecken & Adams (1953) and Vander Eecken (1959).

As to how far these anastomoses are able to fill the vascular bed of an occluded artery soon after its occlusion is not clear. The experiments described in this paper were designed to elucidate this problem in the dog. The passage of blood through an area of the dog hemisphere was observed by injecting ^{32}P-labelled erythrocytes into the carotid circulation, and monitoring their transit through the cortex by a probe counter before and after occlusion of middle and anterior cerebral arteries.

METHODS

Twenty dogs unselected as to age or sex were used. Their weights ranged from 16 to 34 kg, and the majority were greyhounds. This breed has a skull of constant configuration with a low zygoma and small mandibular coronoid process, which renders them particularly suitable for skull dissection.

The dogs were anaesthetized by intravenous pentobarbitone sodium in an initial dose of 25–33 mg/kg body weight, supplemented as necessary. The blood pressure was recorded continuously from the cannulated femoral artery.

Red cell labelling. Red cells were labelled by the method described by Mollison, Robinson & Hunter (1958), to give a final activity when suspended in saline of 40 μc/ml. 0·2 ml. of this solution was diluted to 0·5 ml. with normal saline immediately before use.

Preparation of the neck. The trachea was cannulated, and, after the area of the right carotid sinus was exposed, the carotid sinus nerve, the occipital, internal carotid and ascending pharyngeal vessels were divided. Division of the occipital artery ensures that material injected into the external carotid will not pass into the basilar arterial system via the occipitovertebral anastomoses. As differentiation between internal carotid and occipital vessels is sometimes difficult (Verney, 1947), both were divided. Under normal circumstances much of the blood to the dog hemisphere is carried by the arteria anastomotica and other communications between the internal and external carotid vessels at the base of the skull (Batson, 1944).

A small polythene cannula with total dead space 0·1 ml. was introduced into the lingual

artery so that the tip of the cannula lay at that vessel's junction with the external carotid. Injections of 0·5 ml. of the labelled erythrocyte suspension were made through this cannula from a tuberculin syringe, as rapidly as possible. After each injection the cannula was flushed out and filled with a solution of heparin (500 u./ml.).

Preparation of the head. An inverted U-shaped flap was made, based on the zygoma and extending upwards to the mid line, and from the orbital margin to the anterior margin of the pinna. The temporal muscle was elevated posteriorwards from the skull and partially divided along the zygoma, but not excised. Opening the mouth during this procedure facilitates a low dissection of the muscle (Jewell & Verney, 1957). A wide craniectomy was made from near the mid line to the base of the skull, the frontal air sinus being extensively opened anteriorly. The dura was opened by a U-shaped flap based anteriorly, with division of the branches of the meningeal arteries. Tracing of the flow of labelled erythrocytes through the cerebral cortex was accomplished by using a probe-pattern Geiger counter (20th Century Electronics Type N. 1*b*) of 3 mm external diameter, introduced some 17 mm obliquely to underlie the cortex of the suprasylvian gyrus, so that the depth of the centre of the counter tip was about 5 mm from the pial surface. The active area of the counter is 1 cm long and begins 7 mm from the counter tip. The output of the probe passed to a small cathode follower, and thence to a rate-meter and Evershed pen recorder. A paper speed of 12 in. (30 cm)/min was used throughout.

Dissection of basal vessels. Gentle retraction of the pyriform lobe with a broad spatula enabled the optic nerve, internal ophthalmic artery, and terminal internal carotid artery to be seen. At this stage the origins of middle and anterior cerebral arteries from the carotid were usually concealed by the arachnoid of the basal cistern. Coagulation and division of the internal ophthalmic artery enables the arachnoid to be breached over the olfactory tract and separated backwards by the tip of a fine sucker, displaying the junction of the posterior communicating and internal carotid arteries and the origin of middle and anterior cerebral arteries, with the proximal millimetre or so of their course.

Interruption of the blood flow in the anterior and middle cerebral arteries was performed in two ways. In some experiments the anterior cerebral artery was coagulated about 1 mm from its origin, that is, proximal to the anterior communicating artery, over a length of 1–2 mm, and divided. The middle cerebral artery was coagulated or clipped 1 mm from its origin, and excised as far as its proximal bifurcation in the sylvian fissure with diathermy coagulation of its central branches (Rosomoff, 1957). In other experiments a silver clip was applied to each vessel 1 mm from its origin from the internal carotid and each clip touched momentarily with a diathermy electrode. This method produces reliable occlusion of the vessels.

Perfusion technique. In order to confirm the vascular details as seen at operation, and to obtain information about any areas to which the vascular supply might have been markedly reduced, the head was perfused with a carmine gelatine mass.

At the close of each experiment, complete intradural haemostasis was assured, and the dura was closed with a running suture of fine silk. A few loose stitches then held the temporal muscle back in place. By this means intracranial pressure was re-created at least in part, and the danger of rupture of the exposed area of brain during perfusion was avoided. The dog was killed by clamping the ascending aorta, and the head perfused under constant pressure of 150 mm Hg from the aortic arch, the brachial arteries being tied in the axillae. The right atrium was then opened. The vessels were cleared by perfusion with 1200 ml. of 3·8% citrate solution, and then filled with 1200 ml. carmine gelatine solution containing 2·5% gelatine. The viscosity of this solution at 37° C was 75% of that of blood. During perfusion the gelatine remained in a water-bath at 37° C and passed to the animal through a short length of lagged tubing.

Following perfusion the head was severed, and the entire vault of the skull removed. The dura on both sides was widely opened and the head, with the brain *in situ*, placed in 10%

formalin solution. After 48 hr the brain was removed and transferred to 20% formalin for a further 24 hr. The basal vessels were then examined in detail, and sections made as necessary with a brain knife.

RESULTS

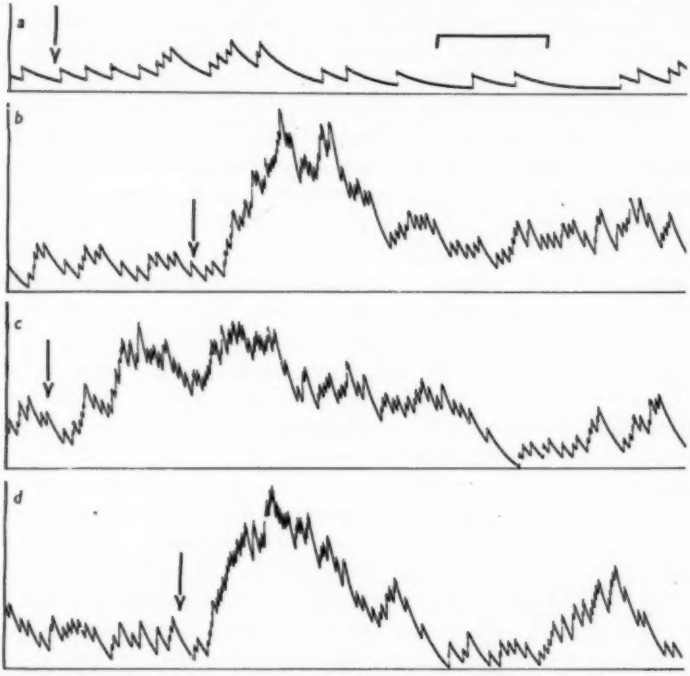
Characteristics of the recording system

When the labelled red cells were injected into the external carotid artery, a steep rise in radioactivity, as shown by a sharp deflexion of the pen, was recorded from the ipsilateral cortex. The latency of this activity was constant, about 1·25 sec (Text-fig. 1*b*). This is considerably shorter than the circulation time from mid common carotid (looped) to ipsilateral retina of 3–4 sec, obtained by Verney (1947) using a dye method. The difference is probably due to the shorter distance under observation in the present experiments, and to the fact that the counter records the presence of isotope in the larger pial vessels, whereas the dye used by Verney could not be viewed by the ophthalmoscope until it appeared as a blue flush in the capillaries of the retina. The deflexion was sustained for a duration which varied from animal to animal, between 9 and 13 sec. The characteristics of the recording system, however, are such that while a rise in activity produces a response with infinitesimal latency, the return of the pen from a position of deflexion is rather slow. This is due to the long integration time necessary in the amplification stage to give an adequate response from a rather insensitive counter at very low levels of activity. Therefore the total time of deflexion does not represent the actual transit time of activity in the cortex, although it reflects this time fairly accurately.

With repeated injections the height and duration of the deflexion remain approximately constant in any one animal, provided the activity, speed and volume of injection are the same. An experiment to illustrate this is shown in Text-fig. 1. At *b* and again 12 min later at *d* the same amount of labelled red cells was injected into the right external carotid artery. Apart from small differences in the shape of the deflexion, which represent the normal variation obtained with this method, the two deflexions were similar in height and duration, and the latency was the same for both. This similarity was obtained although during the 12 min interval labelled red cells were injected into the left external carotid artery whilst the right common carotid artery was compressed. The deflexion produced by this injection is shown at Text-fig. 1*c*. At the beginning of this experiment such an injection was made without compression of the ipsilateral common carotid artery, producing only a small effect, as is shown at Text-fig. 1*a*. Even this small effect was exceptional, for in other experiments such an injection did not produce any deflexion.

With the speed and volume of injection unaltered an increase in the activity of the red-cell suspension resulted in greater deflexion, as is shown

in Text-fig. 2. The duration of the deflexions increased, despite the fact that the volume and speed of injection remained the same. This does not represent an increase in the time of transit of red cells through the cortex, but reflects the fact that with increased activity, because of the properties of the amplifier, it requires a greater time for the pen to return to its initial

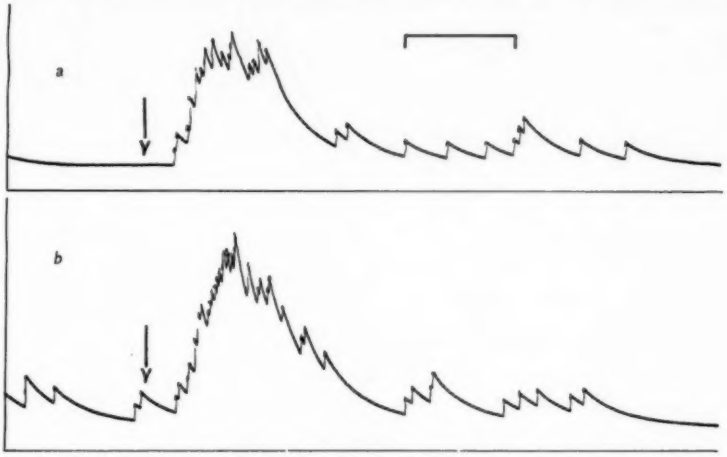


Text-fig. 1. Records of radioactivity obtained from the right suprasylvian cortex of a dog. At each arrow injection of 8 μc ^{32}P-labelled red cells was made, in *b* and *d* into the right external carotid artery, in *a* into the left external carotid artery, and in *c* into the left external carotid artery with the right common carotid artery compressed. Time marker (in *a*), 5 sec.

level. As the height of the deflexion obtained with any given activity varied from dog to dog, it was not possible to predict the height of deflexion with any given activity. On the other hand, because of the cumulative background rise it is undesirable to give repeated injections and therefore it is not possible to standardize height and dose in a given animal. Thus subsequent records can be interpreted qualitatively only.

Experiments were carried out to determine the range, and hence the structures, from which counts could be recorded with the counter used,

whose sensitivity, because of its small size and rather thick wall, is not high. The maximum range in tissue of β particles from ^{32}P is about 7·8 mm (Faires & Parks, 1958). Tests *in vitro* with brain slices, however, showed that at the order of activity of the erythrocyte suspension before dilution (40 μc/ml.) complete extinction of counts was achieved by interposing between the counter and the source a brain slice of 5 mm thickness. This



Text-fig. 2. Records of radioactivity obtained from right suprasylvian cortex of a dog. At the arrow in *a* 6 μc, and in *b* 8 μc, of ^{32}P-labelled red cells were injected into the right external carotid artery. Time marker, 5 sec.

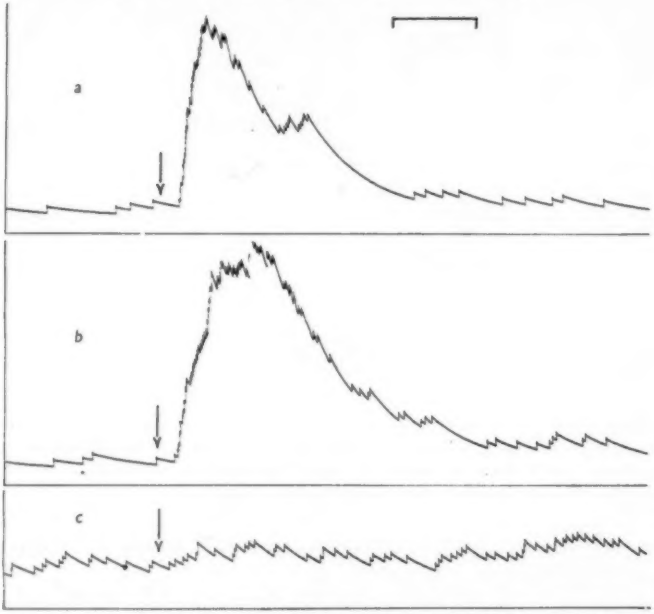
restricts the area from which the counter will record to overlying or neighbouring cortex.

From Pl. 1 *d*, *e* and *f*, it will be seen that the distance between the active area of the counter and the nearest area of cortex supplied by the anterior cerebral artery, the deep pericallosal sulcus, is about 1 cm. There is thus no possibility that the passage of radioactivity through this cortex would be recorded. On the other hand, as shown in Pl. 1, the distance between counter and basal ganglia is very close to the critical value of 5 mm. For all experiments in which the middle cerebral artery was occluded, however, this was of no significance, since the normal blood supply of these areas is from the lateral striate arteries, which arise from the middle cerebral artery distal to its point of occlusion.

Occlusion of anterior and middle cerebral arteries

Occlusion of the anterior cerebral artery by clip (three dogs) or by diathermy (two dogs) did not change the latency of the deflexion. This is

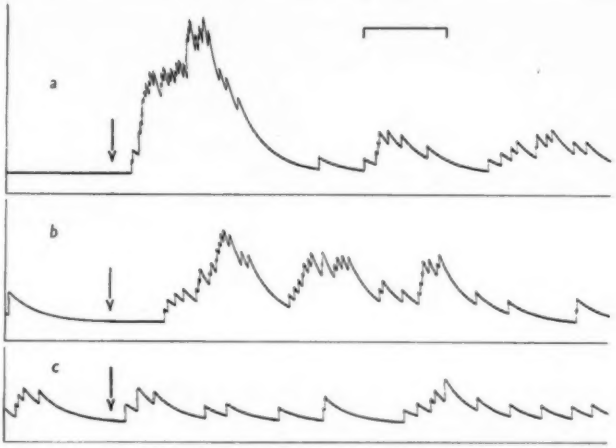
shown in Text-fig. 3, in an experiment in which occlusion was effected by clip. After occlusion, however, there was a perceptible lengthening of the deflexion, reflecting a prolongation of the time during which activity remained in the area of the counter, in other words, a slowing in the blood flow through the overlying cortex. Subsequent occlusion of the middle cerebral artery caused disappearance of the deflexion. The irregularities shown in Text-fig. 3*c* are not significantly above background.



Text-fig. 3. Records of radioactivity obtained from the right suprasylvian cortex of a dog. At each arrow injection of 8 μc ^{32}P-labelled red cells was made into the right external carotid artery, in *a* with the cerebral circulation intact, in *b* with the right anterior cerebral artery occluded, and in *c* with both the right anterior cerebral artery and the right middle cerebral artery occluded. Time marker, 5 sec.

Occlusion of the middle cerebral artery without prior occlusion of the anterior cerebral artery (eight dogs) caused pronounced changes in the form of the deflexion. This was to be expected since the counter was positioned in a region conventionally regarded as within the middle cerebral field of supply. The records of a typical experiment are shown in Text-fig. 4. At *a* is shown the deflexion obtained with the circulation intact. After occlusion of the middle cerebral artery at *b*, the latency of the deflexion is increased, the arrival of activity is more gradual, and the transit of activity significantly prolonged. Height and form of the residual

deflexion varied from animal to animal, but latency and transit time were always prolonged. The wave form obtained after occlusion of the middle cerebral artery showed considerably greater variability than that after occlusion of the anterior cerebral artery. The variations were independent of the method of occlusion. At *c* is shown the effect obtained on subsequent occlusion of the anterior cerebral artery as well. The wave is abolished.

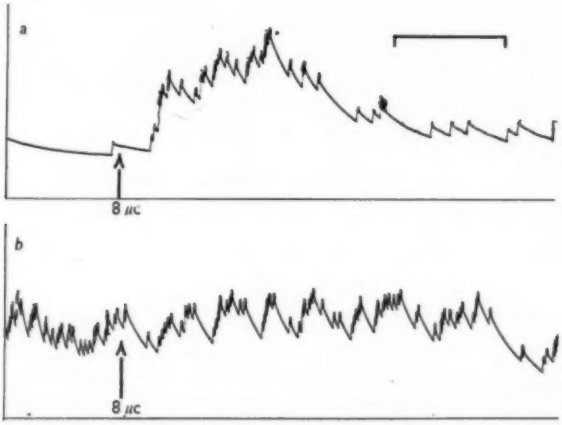


Text-fig. 4. Records of radioactivity obtained from the right suprasylvian cortex of a dog. At each arrow injection of 8 μc ^{32}P-labelled red cells was made into the right internal carotid artery, in *a* with the cerebral circulation intact, in *b* with the middle cerebral, and in *c* with both anterior and middle cerebral arteries occluded. Time marker, 5 sec.

In one dog (Text-fig. 5) occlusion of the middle cerebral artery alone was followed by disappearance of the deflexion obtained, after carotid injection, from the observed cortex. Subsequent dissection revealed that in this dog the right and left anterior cerebral arteries were filled from the left internal carotid, the right anterior cerebral between the anterior communicating artery and the right internal carotid artery being vestigial.

The fact that in all other experiments the deflexion did not disappear with occlusion of the middle cerebral artery alone suggests that the residual and varying activity recorded was conveyed by anastomoses in the leptomeninges between anterior and middle cerebral arteries. This suggestion is supported by the results of the experiment shown in Text-fig. 6. The deflexion with intact circulation is shown at *a* and after occlusion of the middle cerebral artery at *b*. Between *b* and *c* a parasagittal incision about 1 cm from the mid line and 6 cm long was made through the cortex over the whole length of the craniectomy. All the vessels of the pia and

cortex were divided and coagulated in the process, so that many anastomotic vessels conveying blood from the anterior cerebral artery to the area of the counter were interrupted, although it was impossible to divide them all, especially those far anterior. The deflexion recorded when labelled cells were injected after this incision is shown in Text-fig. 6*c*. There is a further delay in arrival of activity and a further attenuation of the deflexion, which is long drawn out. Finally, when the anterior cerebral artery was occluded as well, injection of labelled cells no longer gave any deflexion (*d*).

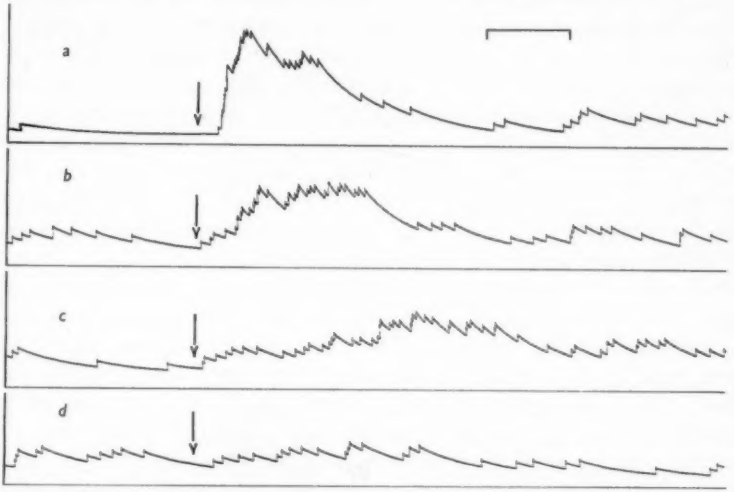


Text-fig. 5. Records of radioactivity obtained from the right suprasylvian cortex of a dog. At each arrow injection of 8 μc ^{32}P-labelled red cells was made into the external carotid artery, in *a* with the cerebral circulation intact and in *b* with the middle cerebral artery occluded. Time marker, 5 sec.

Additional observations of functional anastomoses between the branches of the middle cerebral artery and neighbouring vessels were as follows. In the course of operative excision of the middle cerebral artery, free bleeding from the divided distal end was invariably apparent. Further, at the end of several experiments when the middle and anterior cerebral arteries of one side had been divided, section of any small artery in the pia over the convexity of the hemisphere produced a free flow of blood. This blood must have been conveyed into the pial network either by the anterior cerebral artery of the other hemisphere, which joins the occluded anterior cerebral artery by way of the anterior communicating artery beyond the point of occlusion, or by anastomoses in the leptomeninges from the ipsilateral posterior cerebral artery.

Jewell & Verney (1957) gained the impression that other extracranial vascular occlusions may also influence the distribution of blood from the

basilar and carotid arteries within the skull. Observations made in the course of one experiment confirmed this impression. During the preparation of the neck, it was found that the right occipital artery was particularly large and of a calibre greater than the continuing external carotid artery. It was ligated along with the internal carotid and ascending pharyngeal vessels. On injection of labelled red cells into the right external carotid circulation, the customary deflexion was not obtained from the counter in the right hemisphere, but when the left common carotid was then temporarily occluded such an injection gave a deflexion of the normal



Text-fig. 6. Records of radioactivity from the right suprasylvian cortex of a dog. At each arrow injection of 8 μc ^{32}P-labelled red cells was made into the right external carotid artery, in *a* with the cerebral circulation intact, in *b* with the right middle cerebral artery occluded, in *c* after dividing many leptomeningeal anastomoses between right middle and anterior cerebral arteries, in *d* with the right anterior cerebral artery occluded as well. Time marker, 5 sec.

size and latency. Dissection of the head of this animal showed that both occipital arteries were unusually large. The vessels of the circle of Willis, the internal carotid arteries and the anastomotic rami appeared, to naked eye examination, of normal calibre, but both external carotid arteries were clearly reduced in size. It seems likely that in this animal the occipital arteries via the occipitovertebral anastomoses contributed considerably to the circle of Willis, and that after occlusion of the right occipital artery the flow to the observed area of the right hemisphere came largely from the left side, so that blood passed freely from the right external carotid artery to the cortex only when the left common carotid was temporarily occluded.

Perfusion of the brain

The occlusion of both the middle and anterior cerebral arteries of one hemisphere by clip or diathermy generally reduced the intensity of perfusion of the cortex of the parietal and pyriform lobes, and of the insula. This reduction varied from animal to animal. In only two animals was it extensive, usually it was slight, and sometimes no difference was detectable between operated and non-operated sides.

There were pronounced differences in the extent to which the basal ganglia on the side on which the two arteries were occluded were perfused. When the middle cerebral artery was occluded by clip near its origin, the deep nuclei were perfused well, little difference being detectable between the operated and non-operated side. On the other hand, after excision of the middle cerebral artery from its origin to its proximal bifurcation perfusion of the basal ganglia on the operated side was invariably poor. Under these conditions the region of maximal reduction in perfusion, where no carmine was detectable, involved the body and greater part of the head of the caudate nucleus, the lentiform nucleus and the amygdaloid nucleus. The most rostral part of the head of the caudate nucleus, supplied from the anterior cerebral artery, was well perfused even when this vessel had been clipped at its origin. These findings are illustrated in representative coronal sections of two dog brains in Pl. 2. The brain of the dog shown at *A* was perfused after occluding the anterior cerebral artery by clip at its origin, the middle cerebral artery being clipped at its origin and excised from just distal to the clip to its proximal bifurcation with diathermy coagulation of its central branches. In section *A* 1 the upper lateral part of the head of the caudate nucleus is pale, while the remainder of the brain shows no difference from the unoperated side. Section *A* 2 is cut more caudally and the caudate nucleus of the operated side is not perfused. In section *A* 3, which is cut more caudally still, the body of the caudate nucleus, the amygdala and lentiform nucleus on the operated side are not perfused. The brain of the dog shown at *B* was perfused after occlusion by clip of both anterior and middle cerebral arteries at their origin. As shown in all these coronal sections the basal ganglia are equally well perfused on the two sides. Thus after excision of the middle cerebral artery the basal ganglia fail to be perfused owing to the complete obliteration of the vessel's striate and anterior choroidal branches, whereas, after clipping, these branches fill with perfusion material by reflux from the pial anastomosis along the main stem of the middle cerebral artery.

There were no differences between the two sides in the thalamus or in more caudal areas, irrespective of the method of occlusion of the vessels. In the hypothalamus, the findings were occasionally confused by operative

trauma, but as a rule no appreciable differences could be detected between the two sides. Both dogs *A* and *B* (Pl. 2) show the appreciable degree of cortical perfusion on the operated side. The recordings of cortical radioactivity had shown the typical findings in both experiments.

DISCUSSION

The results of the experiments described show that after acute occlusion of the middle cerebral artery in the dog, substantial blood flow continues in its field of supply, and that part of this flow comes from the anterior cerebral artery of the same side by anastomoses in the leptomeninges.

The anatomical basis of this system of leptomeningeal anastomoses is well founded. Vander Eecken (1959) has convincingly shown that there exist in mammals end-to-end anastomoses between the branches of the anterior cerebral artery in the intercerebral fissure and the branches of the middle cerebral artery on the convexity of the hemisphere. The extent of these collateral anastomoses appears to regress *pari passu* with the development of cortical convolutions, both phylogenetically and during human foetal and neonatal development, though Vander Eecken is careful to avoid any attribution of a causal relationship.

The arterial supply of the telencephalon of the dog falls into two distinct groups, the surface vessels and the deep branches penetrating the brain. For the surface vessels an effective collateral circulation is assured by the leptomeningeal anastomoses. In the radioactive experiments here described, despite division of a main cerebral artery, blood from the carotid circulation passed rapidly into the cortex normally supplied by the occluded vessel. Even when the carotid circulation of one side was entirely excluded from the supply of one hemisphere, free blood flow could be obtained from the pial vessels on the surface of this hemisphere in any area, and the cortex could be perfused well after the animal had been killed. These results are in accord with the finding of Petersen & Evans (1937) confirmed by Meyer (1958) that, in monkeys, clipping of the middle cerebral artery near its origin does not result in cessation of circulation in the tributaries, although, on microscopic observation, flow is seen to be appreciably slowed.

Conflicting opinions have been expressed about the neurological deficit produced by acute main vessel occlusion in dogs. Thompson & Smith (1951) found no persisting neurological deficit after occlusion of the middle cerebral artery provided a normal arterial blood pressure was maintained. Jewell & Verney (1957), in the course of their localization of osmoreceptors, had occasion, in three dogs, to tie, amongst other vessels, the middle cerebral artery. None of these dogs developed hemiplegia. On the

other hand, Rasmussen (1938) and Rosomoff (1956) invariably produced neurological deficit in dogs after acute middle cerebral occlusion. Analysis of Rosomoff's (1956) results, however, reveals that while all the animals subjected to occlusion of the middle cerebral artery at normal temperature developed infarction in the basal ganglia, less than half showed any evidence of infarction in the cortex.

The brisk and substantial collateral flow demonstrated in the tracer experiments here described probably represents a collateral circulation adequate to maintain the function of cortical cells after the main artery which normally supplies the area has been acutely occluded.

In the deep branches of the cerebral vessels which supply the basal ganglia, there is much less collateral anastomosis than in those on the pial surface. From the perfusion experiments recorded in this paper it can be seen that the lentiform nucleus and the amygdala were perfused only from the deep branches of the middle cerebral artery. The caudate nucleus showed some evidence of a dual blood supply, since after occlusion of the striate branches of the middle cerebral artery, the rostral part of the head of this nucleus was found to be perfused. There was no evidence of any other blood supply to the basal ganglia, however, apart from this small area. Its limited extent shows that collateral function between anterior and middle cerebral fields within the caudate nucleus is not comparable to that between the branches of the same two vessels on the pial surface.

It has been shown in survival experiments that, in the dog, occlusion of the main arterial supply of the basal ganglia is always followed by infarction, although not necessarily by clinical evidence of neurological damage. Thus Rosomoff (1956) demonstrated that hypothermia substantially reduced the neurological deficit produced by his method of occlusion of the middle cerebral artery in which the deep branches of the vessel are divided, but that it did not prevent infarction of the putamen, globus pallidus and amygdala. It is of interest to note that in Rosomoff's experiments the caudate nucleus, shown on perfusion to have a dual blood supply, invariably developed infarction at normal temperatures but not under conditions of hypothermia. This suggests that the pre-capillary and capillary anastomoses within the caudate nucleus, for which evidence has been provided by Pfeiffer (1928) and Cobb (1931), were capable of developing a collateral blood supply between the anterior and middle cerebral fields which could become adequate with the delay in metabolic demand following a period of lowered temperature.

The experiments recorded in this paper were performed in dogs, in which the freely anastomosing nature of the arterial tree outside the skull has long been known. As is shown in the present experiments, this characteristic applies also to the cortical vessels, but preliminary observations in

monkeys indicate that the collateral potential in this species is much less, so that it is not justifiable to apply the results obtained in dogs to other species, particularly to monkeys and man.

SUMMARY

1. A method is described of observing the flow of ^{32}P-labelled red cells through the cerebral cortex of the dog.

2. By this method it has been shown that after acute occlusion of the middle cerebral artery in the dog there is persistent blood flow in the cortex of the middle cerebral field, and that part of this flow comes from the ipsilateral anterior cerebral artery by leptomeningeal anastomoses.

3. By perfusion techniques the profuse collateral circulation of the dog cerebral cortex is confirmed, contrasting with the basal ganglia which are shown to have a poor collateral blood supply.

4. The significance of these findings in relation to the functional effects of acute occlusion of the middle cerebral artery in this species is discussed.

This work was performed during the tenure of a Clinical Research Fellowship from the Medical Research Council. The author wishes to express his thanks to Sir Charles Harington for facilities provided in the National Institute for Medical Research, to Dr W. Feldberg for his interest and to Mr D. Mason, Electronics Division, National Institute for Medical Research, who designed and built the pre-amplifier and rate-meter.

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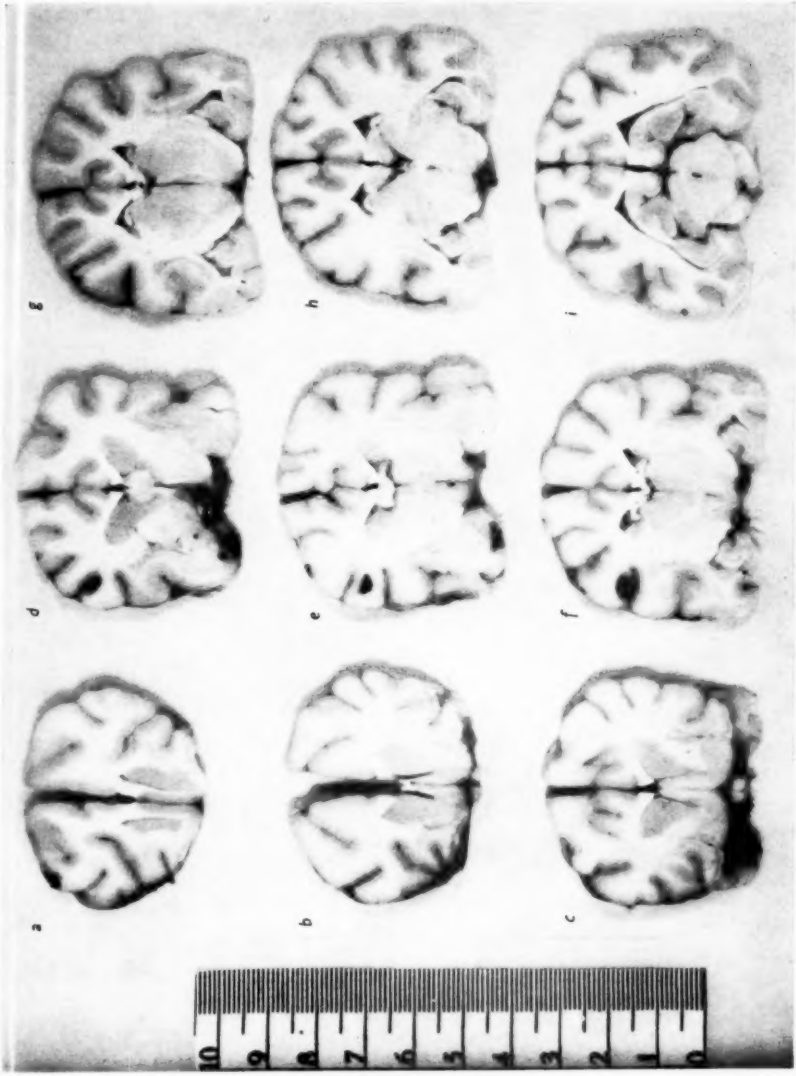
EXPLANATION OF PLATES

PLATE 1

Coronal sections of dog brain after perfusion with carmine gelatine. Records from this experiment shown in Text-fig. 4. The area of insertion of the counter is visible in *d*, *e* and *f*. In *c* a clip is visible on the anterior cerebral artery, and in *d* on the middle cerebral artery. Some operative trauma in the hypothalamus and pyriform lobe seen in *d* and *e*. Scale in centimetres.

PLATE 2

Representative coronal sections of two dog brains after perfusion with carmine gelatine. In *A* 1–3 the middle cerebral artery was clipped at its origin and excised from the clip to its proximal bifurcation with diathermy coagulation of the branches. In *B* 1–3 the artery was clipped at its origin. The artery was effectively occluded. In *A* 3 and *B* 3, the insertion of the counter can be seen. In *A* 3, only the most lateral part of the blackened area was occupied by the counter, the medial part of this area is due to blood staining in the adjacent cortex. Scale in centimetres.



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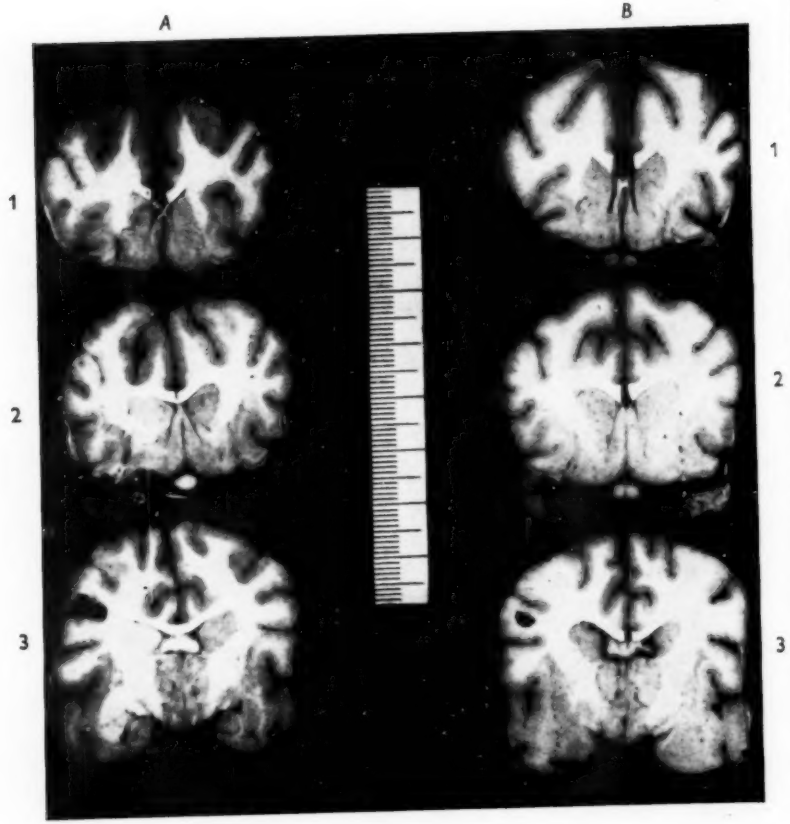

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THE EFFECT OF INITIAL STRIP LENGTH ON THE NORADRENALINE-INDUCED ISOMETRIC CONTRACTION OF ARTERIAL STRIPS

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The resistance to stretch of strips, rings and segments of arteries has been extensively studied since 1880 (Remington, Hamilton & Dow, 1945; Roach & Burton, 1957). Less is known, however, about the effect of changes in the degree of stretch of the arterial wall on its subsequent response to stimuli. Ducret (1931) has shown that the initial degree of stretch of mesenteric arterial rings from a variety of species determines the force of the adrenaline-induced isometric contraction of the ring. This study dealt only with changes in length in the region where the extensibility of the ring was small. The object of the present study was to examine the effect of initial strip length on the isometric contraction of arterial strips over a wider range of length, using noradrenaline instead of adrenaline as the vasoconstrictor. A detailed study of these length–tension relationships of arterial strips promised to be useful in establishing the position of arterial muscle contraction with respect to muscle contraction in general. The findings of some of these experiments have already been briefly described (Speden, 1958, 1959).

METHODS

Segments of the jejuno-ileal branches of the superior mesenteric artery were obtained from freshly slaughtered sheep and stored in cold Krebs–Henseleit solution until used about an hour later. Excess fat and connective tissue were trimmed from an artery of internal diameter of approximately 1 mm (in the contracted state), and a 1–2 cm length of the artery was slipped on to a 24-gauge stainless-steel syringe needle covered with polythene tubing (Allen & Hanburys Limited, external diameter 1 mm). The steel tube (*a*) shown in Fig. 1*A* and *B* was held by means of a chuck (*b*) inset into the long axis of a $\frac{5}{8}$ in. (16 mm) iron bolt (*c*), with clockwise thread of 12 turns per inch (2/mm). This bolt was screwed horizontally into a brass mount (*d*) clamped in a vice (*e*). A circular cutting wheel, rotated by means of a variable-speed series motor, was lowered on to the polythene (*f*) and a clockwise helical strip cut by screwing in the bolt. The cutting wheel was placed at approximately 45° to the needle's long axis so that the wheel followed the angle of cutting. By this means an arterial strip, 2 mm wide, can be quickly and easily obtained from small arteries. For satisfactory

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cutting the steel needle needs to be carefully centred into the bolt's long axis and the play between the bolt and its mount must be small.

For the isometric recording a 5–10 mm length of the strip was mounted vertically by clamping the ends of the strip between two Perspex clamps. The upper clamp was directly attached, by means of a light-weight chrome-plated jeweller's chain to a 1 cm brass rod extension of the external pin of an RCA 5734 mechano-electronic transducer valve. The valve output was fed into a DC amplifier and, after amplification, recorded simultaneously on a meter and a Southern Instruments pen recorder. For the stress relaxation experiments the time constant of the amplifying system was adjusted to less than 0·04 sec. The lower Perspex holder was fixed to the long limb of an inverted glass L piece, the short limb of which was attached to the vertical movement of a screw-threaded adjustable stand. The strip length

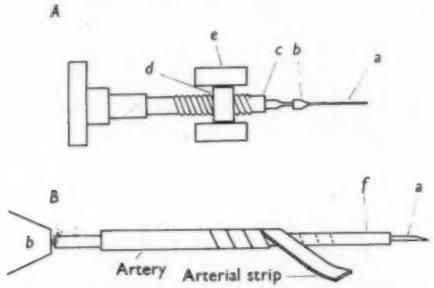

Fig. 1. *A*, The apparatus used for preparing an arterial strip. *B*, the needle part of the apparatus in more detail. For description see text.

was changed by raising or lowering the holder by means of this stand, in which play was negligible. For the slow continuous changes in length a motor-driven pulley system, attached to the stand, was used. Length changes were measured to an accuracy of $\pm 0{\cdot}2$ mm. In the preliminary experiments nylon or cotton thread, tied to the ends of the strip, was used. This introduced large compliance errors and was replaced by the above attachments.

The mounted preparation was transferred to a constant-temperature bath. The outer bath, filled with water, was maintained thermostatically at 37·5° C. The inner bath (25 ml. capacity), in which the preparation was immersed, contained a Krebs–Henseleit solution of the following composition (mM): NaCl 118, KCl 5·32, NaH_2PO_4 1·54, $MgSO_4.7H_2O$ 1·19, $NaHCO_3$ 24·9, $CaCl_2$ 2·53, dextrose 5·6, ethylene diamine tetraacetic acid (disodium salt) 0·01; pH 7·2–7·4. The distilled water used was first passed through a column containing a mixed anionic-cationic exchange resin (Biodeminrolit, Permutit Co. Limited, London). NaCl, KCl, NaH_2PO_4, $MgSO_4.7H_2O$, and $NaHCO_3$ were then added and the solution was saturated with a gas mixture of 95% O_2 and 5% CO_2 before addition of the remaining components. Ion-exchange-treated distilled water and the chelating agent were used so that trace concentrations of copper and iron ions, which catalyse the oxidation of catechol amines, could be reduced. For the same reason all metal components of the cutting equipment with which the strip was likely to come in contact were coated with shellac. The Krebs-Henseleit solution flowed from a reservoir through a polythene heat-exchange coil lying in the outer bath, before entering the inner bath. A gas mixture of 95% O_2 and 5% CO_2 was continuously bubbled through the solution in the inner bath.

The noradrenaline (L-noradrenaline bitartrate, monohydrate) was diluted to the required concentration with NaCl (0·9 g/100 ml.) containing ethylene diamine tetraacetic acid (disodium salt, 0·01 mM). The drug was injected directly into the inner bath in a volume not exceeding 1% of the total bath volume. The noradrenaline was removed by washing away

the inner bath solution with sufficient fresh solution to dilute the noradrenaline to below its minimum effective concentration. Such washing did not lower the bath solution temperature more than 1° C. When no noradrenaline stimulation was used the inner bath solution was continuously changed at a rate of 2·5 ml./min. Noradrenaline concentrations are expressed as weight of the free base per millilitre.

The shortest length attained following noradrenaline stimulation (1–10 μg/ml.) was used as a *reference length* (here termed the *pre-shortened length*). This was most conveniently estimated by stimulating with noradrenaline and, after allowing the strip to shorten freely, taking up the slack by progressively stretching the strip until a small tension (0·1–0·2 g) appeared. The drug was then washed out.

For the histological investigation each strip was mounted, unstretched, on Bristol board and then placed in buffered formaldehyde fixative. After blocking and sectioning the sections were stained with either Van Gieson-celestin blue or with Gomori-haematoxylin orange G.

RESULTS

A number of preliminary experiments were carried out to determine the most suitable conditions for examining the effect of initial length on the noradrenaline contraction. As the contraction is superimposed on the resting state it was first necessary to study the properties of the resting muscle. The effect of rate of stretch on the tension developed, and the reproducibility of the length–resting-tension curves, were therefore studied.

Length changes in resting arterial strips

The tension changes produced by stretching resting arterial strips depended on the rate of stretching.

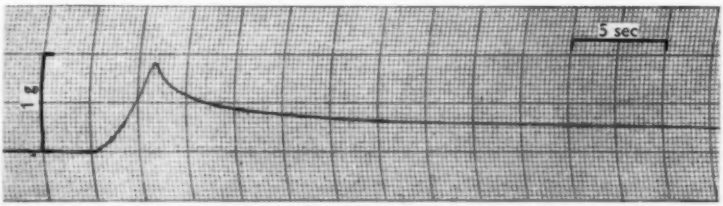

Fig. 2. Stress relaxation curve for the sheep mesenteric arterial strip. The strip was stretched from the pre-shortened length of 4·4–8·0 mm. Residual tension 10 min after end of stretch 0·20 g; strip weight 5 mg; vertical bar, 1 g; time marker, 5 sec.

Rapid changes in length. When a strip was rapidly stretched and held at the new length, the tension rose during stretch and then fell, at first rapidly then more slowly (Figs. 2, 3). This decay of tension following rapid stretch (stress relaxation) is characteristic of viscous-elastic materials. The time course of the tension decay was examined in six strips by first pre-shortening the strip, then, 10 min after washing out the drug, rapidly stretching the strip by screwing down by hand the vertical

movement of the adjustable stand. The rate of stretching was approximately 2 mm/sec and was kept as constant as possible. For small stretches from the pre-shortened length the tension decay was complete, but after larger stretches, which gave curves suitable for analysis, the decay of tension was incomplete. As a means of comparing the results, the residual tension

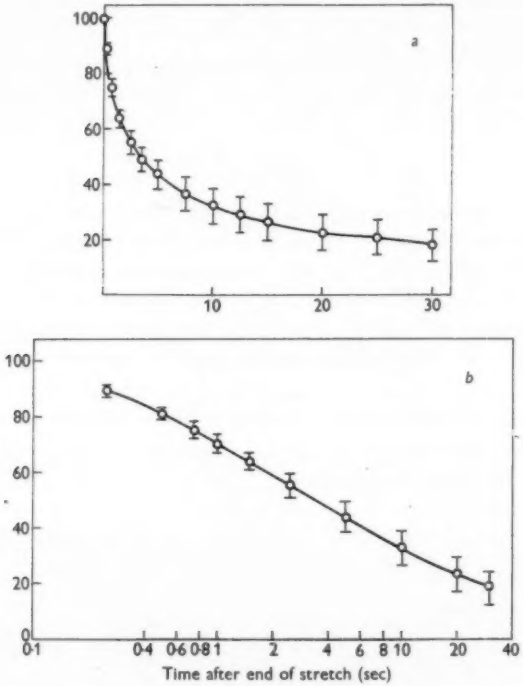

Fig. 3. Percentage decay of the transient tension, following rapid stretch from the pre-shortened length, in the resting sheep mesenteric arterial strip; *a*, on a linear time scale; *b*, on a logarithmic time scale. Each point is the mean (±S.D.) obtained from 12 decay curves, 2 from each of 6 preparations. The mean % increase in length following stretch was 78±22% (S.D.).

remaining 10 min after stretch was subtracted from the total tension at any one time and the difference expressed as the percentage of the total amount of tension decay in 10 min. The mean curve from twelve such stress–relaxation curves is shown in Fig. 3*a*. When the data in Fig. 3*a* were plotted on a logarithmic time scale the decay of the percentage of the total transient tension was found to be linear over several log. cycles of time (Fig. 3*b*). This linear relationship has also been observed in the

human umbilical and the dog carotid artery (Zatman, Stacy, Randall & Eberstein, 1954).

Slow continuous changes in length. With slow continuous stretch or release no rapid transient tension changes occurred. At the various lengths during the stretch and the release the opposing tension was measured to give the corresponding length–tension curve (Fig. 4). The curves obtained from a series of repeated stretches and releases could not be superimposed, each curve being displaced to a greater length, relative to tension, com-

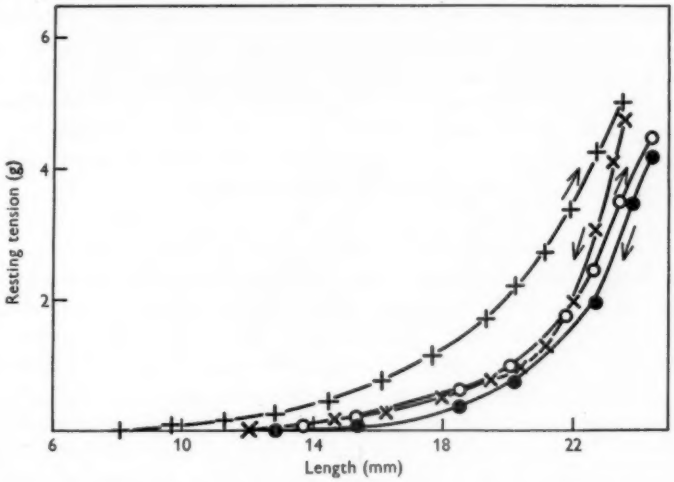

Fig. 4. Length–resting-tension curves of the sheep mesenteric arterial strip. Resting tension on first stretch (+), first release (×), second stretch (○) and second release (●). Rate of length change 1·6 mm/10 min, strip weight 7·7 mg, pre-shortened length 8·1 mm.

pared to the preceding curve. This shift to greater length was most marked between the first and all subsequent curves. If, however, the strip was stretched until a tension of 4–6 g was maintained before determining the curves, the difference between repeated length–tension curves over the range of length studied was small. Similar differences between repeated length–resting-tension curves have been observed in the aortae of the cow, dog and man (Krafka, 1939; Remington *et al.* 1945). These authors attributed this to incomplete smooth-muscle relaxation. Additionally, irreversible stretch of non-contractile elements in the arterial wall may partially explain the present findings. The effect of these factors on the isometric contraction was minimized by stretching the strip before the start of the experiment.

Response to noradrenaline

The tension developed on noradrenaline stimulation increased from a minimum detectable contraction with 0·01–0·04 μg/ml. up to a maximum with 1–10 μg of the drug/ml. After stimulation with noradrenaline 1–5 μg/ml., the tension at first developed rapidly over a period of 20–40 sec then more slowly, reaching a maximum in 1·5–10 min (Fig. 5). This contraction, which was usually maintained, was reversed over a period of 5–10 min following washing. With the lower drug concentrations the shape of the response could take a number of forms, being either maintained, like the responses to the higher noradrenaline concentrations, not maintained, or inducing rhythmic contractile activity. Similar variations in the shape of the adrenaline responses of mesenteric arteries have been observed by many workers (see Burgi, 1944). It was found that a relatively consistent response, over a period greater than 2 hr, was obtained only with maximal or near-maximal noradrenaline stimulation.

Active and resting isometric-tension–length curves

The length–isometric-tension curves on both stretch and release were examined fifteen times in twelve strips with maximal or near-maximal noradrenaline stimulation. Two methods were used. The better, with rapid length changes, made use of the previous observations that following rapid stretch of a resting arterial strip there was about an 80% decay of transient tension in 25 sec (Fig. 3) and that within 5 min the tension had reached a reasonably steady value. The strip was first stretched until a resting tension of 4–6 g was maintained before determining the pre-shortened length; then, starting from the pre-shortened length, the strip length was increased stepwise, an interval of 7 or 9 min being allowed after each step before measuring the resting tension and stimulating with noradrenaline. The experiment was then repeated by progressively decreasing the length. This procedure gave both the active and resting-tension–length curves (Fig. 6).

The other method, in which the length was changed slowly and continuously, was somewhat unsatisfactory, as the modification of the tension developed on stimulation due to the continuous change in length and resting tension led to appreciable errors when the resting tension was large and changing rapidly.

The effect of initial length on the force of the isometric contraction induced by maximal noradrenaline stimulation is shown in Fig. 5 and presented graphically in Fig. 6 along with the resting-tension curves. These show that isometric contraction induced by noradrenaline increased with elongation from a barely detectable value at the pre-shortened length

up to a maximum, then decreased with further increases in length. The curves obtained either on stretch or release were similar, the maximum isometric contraction being developed at a length 2·5–3 times the pre-shortened length. At this length an appreciable resting tension was present (Fig. 6). These results were typical of nine of the experiments. The

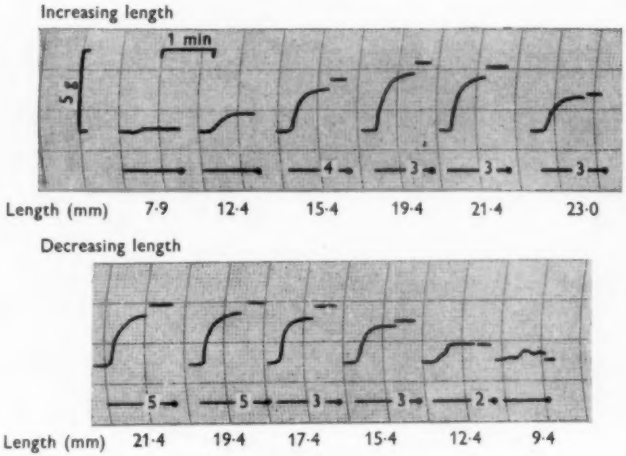

Fig. 5. The effect of initial length on the force of the isometric contraction of the sheep mesenteric arterial strip, with maximal noradrenaline stimulation (5 μg/ml.). ——, exposure to noradrenaline, the numeral giving the time (min) during which the chart was stopped. ●, chart stopped and drug washed out. The interval between contractions was 20 min with the length being changed 10 min before the next contraction. Pre-shortened length 7·9 mm, strip weight 3·9 mg. The figure is a trace from the original record.

remaining six differed only in that the upper limit of the recording equipment (10 g) was reached before a maximum was attained.

Over a length range of 1·5–2·0 times the pre-shortened length the isometric contractions induced by smaller doses of noradrenaline (0·1–0·4 μg/ml.) were also correspondingly influenced by the strip length. In all of seven preparations large increases in length within this range increased the isometric contraction. These experiments tended to be influenced by changes in sensitivity of the strips, usually a loss of sensitivity, but the change in contraction was so great (and in the opposite direction to the change in sensitivity) that the result was clear-cut.

The results would appear to be not greatly affected by inherent tone in the strip. No fluctuating level of spontaneous tone was observed, nor did $NaNO_2$ (100 μg/ml.), which relaxes arterial muscle (Burgi, 1945; Furchgott

& Bhadrakom, 1953) cause any marked relaxation. Thus, after a resting strip had been stretched until a tension of 4–6 g was maintained and then released to a tension of 0·5–1·6 g, the addition of $NaNO_2$ induced a fall of tension of 0·1 g or less (in five preparations). In two strips $NaNO_2$ was added after the completion of the length–resting- and active-tension experiment and had the same small effect.

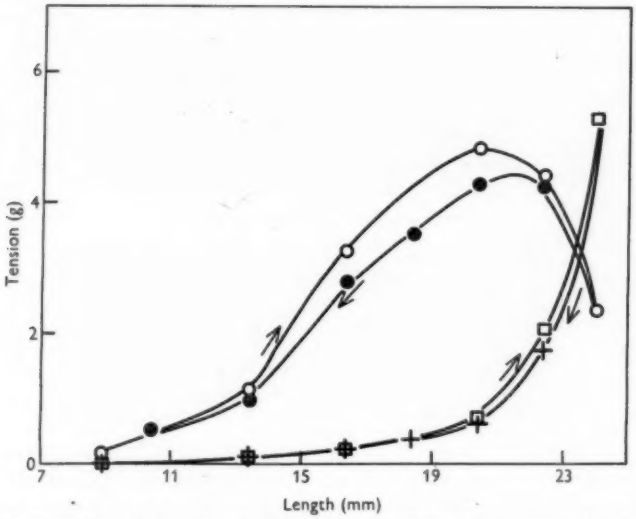

Fig. 6. Length–resting- and active-tension curves of the sheep mesenteric arterial strip. The active tensions developed on noradrenaline stimulation 10 min after an increase in length (○), and 10 min after a decrease in length (●), were obtained from Fig. 5. Resting tensions on stretch (□) and on release (+) were measured 1 min before stimulation.

The observed increase in force of isometric contraction with increasing length could in part be due to re-alignment of obliquely lying muscle fibres into the long axis of the strip (thus leading to a more favourable situation for exerting force). While it was not possible to obtain any quantitative estimate of this, the following histological evidence suggested that re-alignment of muscle fibres was not an important factor. Transverse and longitudinal sections were obtained from seven strips which had been mounted unstretched and were subjected only to the stretching unavoidably associated with the preparing of the strip. In all the longitudinal sections the majority of the nuclei had the typical elongated shape found in a muscle fibre which had been cut longitudinally, whereas in all the transverse sections the shape of the majority of the nuclei was typical of a muscle fibre cut transversely (Fig. 7). The muscle fibres appear, then, to

be mainly aligned in the long axes of the strips. Since these strips were cut obliquely from the intact artery, in which the muscle fibres are arranged mainly circularly about the lumen of the vessel, the muscle fibres must have been re-orientated during the preparation of the strip.

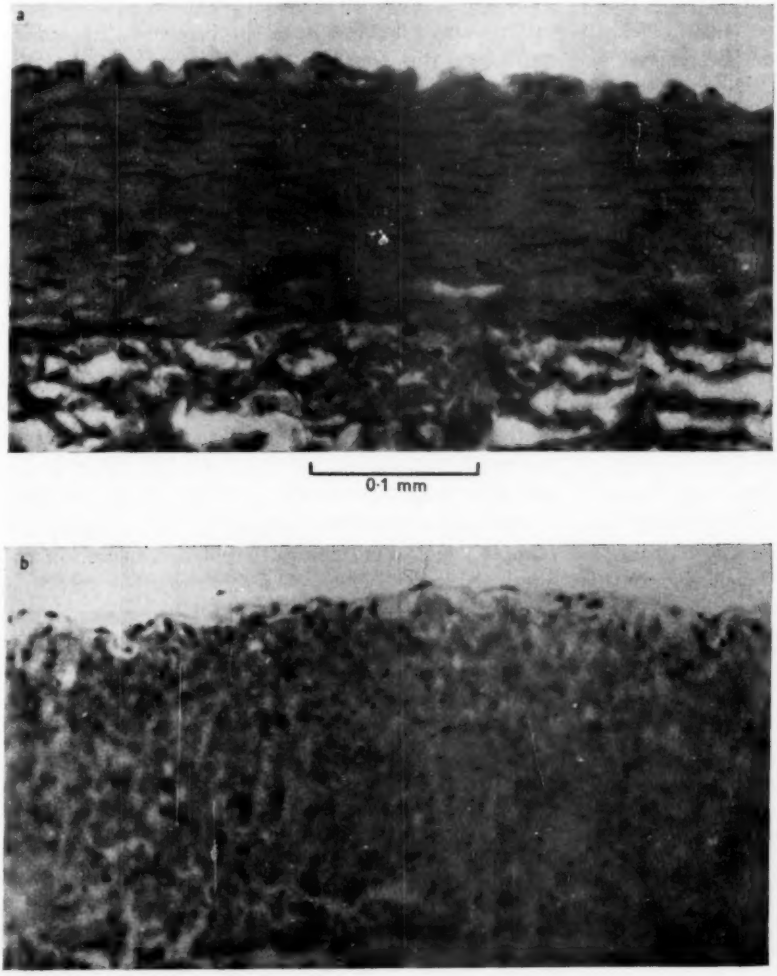

Fig. 7. Muscle fibre orientation in the sheep mesenteric arterial strip: *a*, longitudinal section; *b*, transverse section (from a different strip). Stain, Van Gieson-celestin blue.

DISCUSSION

The force of the isometric contraction of the sheep's mesenteric arterial strip induced by noradrenaline was found to depend on the initial length of the strip. This supports earlier observations obtained with adrenaline stimulation (Ducret, 1931). Although Alexander (1954) did not comment on this, his length–tension curves derived from the pressure–flow curves of the relaxed and contracted dog thoracic aorta also suggest that the active tension developed on adrenaline stimulation depends on the initial length of the muscle.

The more detailed nature of the present results allows a comparison to be made between arterial-muscle contraction and muscle contraction in general. As with frog skeletal muscle (Aubert, Roquet & van der Elst, 1951), uterine muscle (Csapó & Goodall, 1954), intestinal muscle (Brocklehurst, 1926), cat nictitating membrane (Hampel, 1934), dog penis retractor (Winton, 1926) and molluscan muscle (Abbott & Lowy, 1958), the isometric contraction of the arterial smooth muscle increased with elongation up to a maximum, then decreased with further length increases. Compared to skeletal muscle, however, arterial muscle can develop an active tension (on noradrenaline stimulation) over a remarkably wide range of length, a detectable contraction being present at 35–40 % of the length at which a maximum contraction develops. Skeletal muscle develops no tension at the equivalent short length (Aubert *et al.* 1951). Unlike skeletal, molluscan and uterine muscle an appreciable resting tension was present at lengths shorter than that at which a maximum isometric contraction was developed. In this respect arterial muscle more nearly resembles intestinal muscle and the muscles of nictitating membrane and dog penis retractor.

The dependence of the force of the isometric contraction on initial length of muscle appears to be a fundamental property of many contractile tissues. It is tempting then, to suggest that were this dependence to hold, not only for arterial muscle *in vitro* but also for arteriolar muscle *in vivo*, it would be likely that the length of the muscle in the blood vessel wall would play some part in the mechanisms controlling blood pressure.

SUMMARY

1. A method of preparing strips from the muscle of the sheep's mesenteric artery is given.

2. The time course of the stress relaxation in the resting strip was found to be linear when tension was plotted against the logarithm of time over at least 2 logarithmic cycles of time.

3. The resting tension which developed at any length depended on the previous history of the strip with respect to stretch.

4. A stable noradrenaline response was obtained only on maximal or near-maximal stimulation (1–10 μg/ml.).

5. The force of the isometric contraction developed on noradrenaline stimulation (1–5 mg/μl.) increased with length of strip up to a maximum, then decreased with further increases in length.

6. These results have been discussed in relation to muscle contraction in general.

I wish to thank Dr E. G. McQueen for suggesting the problem which led to this work and for his continued interest and encouragement; Professor F. H. Smirk and Dr F. N. Fastier for their generous advice and criticism, Mr A. T. Wallis who constructed the apparatus and Dr T. C. Highton for the histological study. I am indebted to the Medical Research Council of New Zealand for defraying the cost of this work and to I.C.I. (N.Z.) for the award of a Fellowship.

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OBSERVATIONS ON VASCULAR AND METABOLIC RESPONSES IN THE BRAIN AFTER ADMINISTRATION OF INSULIN AND GLUCOSE

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The technique of 'internal calorimetry' (Grayson, 1952) has been extended so that both blood flow and metabolic heat production can be measured simultaneously in solid organs (Grayson & Kinnear, 1958). A full examination of the technique with observations on its use in the assessment of carbohydrate metabolism in liver and muscle has been made by Dosekun, Grayson & Mendel (1960).

Since the measurements made by this method are limited to a small volume of tissue surrounding the recorders, it seemed that this technique might well afford a fresh approach to functional relationships within the brain. In the present work measurements were made in the thalamus and three cortical areas—sensory, motor and occipital. This paper describes an investigation into the different responses of these four regions in relation to certain aspects of carbohydrate metabolism.

METHODS

Internal calorimetry. The technique of and principles underlying internal calorimetry depends on the thermo-electric measurement of thermal conductivity. It has been shown elsewhere (Grayson, 1952) that conductivity increment δk (i.e. the thermal conductivity of living tissue less the thermal conductivity of dead tissue) is an approximately linear function of blood flow.

In this work a Cambridge four-channel recording camera was used with two channels converted to temperature recording by the substitution of D'Arsonval units for the standard oscillators. Simultaneous recording could thus be obtained from two separate thermocouples. Four recorders were, in fact, implanted in the brain and a selector switch enabled recording to be made at will from either of two pairs of recorders. The circuit is given in Fig. 1.

The heater leads of all four recorders were connected in series so that a single current source (a 6 V car battery), controlled by the cam-operated switch gear described previously (Grayson & Mendel, 1957), supplied all four recorders simultaneously. Thermal equilibrium levels were thus maintained throughout the experiment in all recorders and were not affected by the selector switch described above.

Operative procedure. The animals used in this work were male and female West African

Green monkeys (Cercopithecus aethiops) of average weight 3·5 kg. Anaesthesia was induced with ethyl chloride and maintained with intraperitoneal pentobarbitone sodium (60 mg/kg body weight). Two trephine holes (18 mm diameter) were made, one exposing the central sulcus and lateral cerebral fissure and the other the occipital lobe.

The thalamic recorder was inserted as follows. In the West African Green monkey the central sulcus is readily recognizable. At its lower end, where it approaches the lateral cerebral fissure, it is joined by a large artery approaching its anterior lip. Insertion of the recorder in the transorbital plane, in the angle between this vessel and the sulcus, to a depth varying slightly with brain size—usually to a depth of 18 mm—brought the tip of the recorder

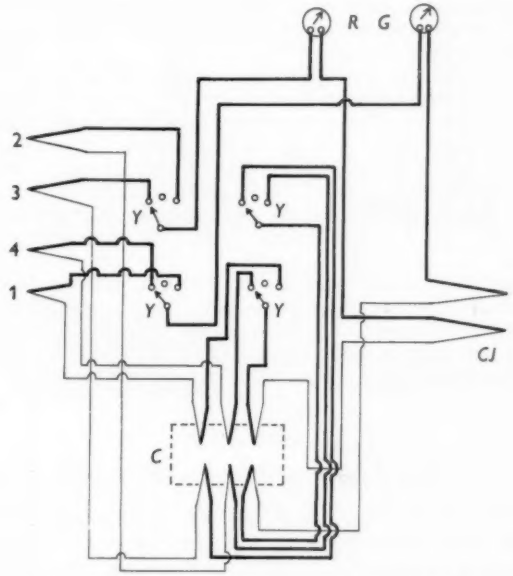

Fig. 1. Circuit diagram of control panel. 2, 3, 4, 1 recording thermojunctions. The Yaxley switch *Y* (Radiospares Ltd.) has three positions. Left brings the thermocouples attached to recorders 3 and 4 into circuit with the two recording galvanometers (*RG*). Centre = open circuit. Right brings thermocouples from recorders 1 and 2 into circuit with the galvanometers. All joints are soldered. No terminals used except at the galvanometers. Cold junctions (*CJ*) mounted in a polyethylene tube near the common carotid artery. *C*, compensating box

deep into the posterior group of thalamic nuclei. The thermocouple itself, which is the real recording point, came to rest in the middle of a bulk of thalamic nuclear tissue. The accuracy of the insertion was checked in every case after the experiment by dissection of the brain, and was found to be reliable in every case.

The cortical recorders were inserted under direct vision in a plane parallel to the surface of the brain, so that the recorder was surrounded by grey matter. Recordings were usually made from the motor cortex, sensory cortex and occipital cortex. After insertion recorders were soldered to the thermo-electric and heating circuits, the skin incision was closed and the head covered with cotton wool.

Insertion of the cold junctions. Since recording was from two circuits simultaneously it was necessary to have two cold junctions. These, made from fine gauge copper and constantan (S.W.G. 30), were mounted together in a fine polyethylene tube which was passed up the antebrachial artery so that the cold junctions came to lie near the origin of the common carotid artery.

Injections were made through an indwelling polyethylene cannula in a femoral vein. Blood sugar samples were taken from the femoral artery and estimated by the method of Nelson (1944).

Determination of metabolic heat production. The concept of 'excess temperature' as an indicator of metabolic heat production has already been described at length. Briefly, the calculation is based upon the recording of lower equilibrium temperature, i.e. the temperature of a heated thermocouple through which a current of 0·200 A^2 is passing (Grayson, 1952). This is read from the photographic trace.

The cold junction was in the stream of blood supplying the brain, and changes in blood temperature due to causes outside the brain were thus automatically allowed for. Under these circumstances changes in lower equilibrium temperature have been shown to be due either to changes in blood flow or to changes in local heat production. The effect of changes in blood flow can be predicted from the record. Changes in lower equilibrium temperature beyond those thus predicted are referred to as 'excess temperature' and are due to alterations in local heat production. The assumptions involved in this procedure have previously been discussed at length (Dosekun *et al.* 1960), as have also the methods of calculation.

Although in the liver it was possible to take the calculation further and make quantitative deductions, the conditions never occurred to make this further extension to the calculation with respect to brain. Accordingly, 'excess temperature' should be regarded as a qualitative index only enabling changes in heat production to be assessed from an arbitrary starting point, but giving no idea of resting or absolute values of heat production.

RESULTS

Blood flow distribution in the brain

Table 1 gives the result of δk measurements in eight monkeys lightly anaesthetized with pentobarbitone sodium. In individual monkeys no consistent pattern of distribution of blood flow as between motor, sensory and occipital cortex could be detected, nor any consistent relation between

TABLE 1. Resting maximum δk values ($\times 10^{-4}$) in the cortex and thalamus in eight monkeys. The values shown are the averages of five successive cycles

		Cortex		
Expt. no.	Thalamus	Motor	Sensory	Occipital
1	1·0	3·0	4·4	3·7
2	3·3	4·8	0·7	1·3
3	1·5	—	1·7	1·6
4	3·6	—	2·2	—
5	2·9	0·6	2·8	—
6	5·1	1·8	2·7	5·1
7	2·1	2·2	2·3	—
8	1·0	—	3·5	—
Means	2·5	2·5	2·5	2·9
S.E.	0·5	0·7	0·4	0·9

the cortical areas and the thalamus. Mean δk values, from all four regions, were similar.

In three monkeys recording from the white matter deep to the parietal cortex was made before measuring cortical flows. The δk values were uniformly low. In one experiment an average δk value of $0{\cdot}6 \times 10^{-4}$ was found, in another $0{\cdot}2 \times 10^{-4}$ and in the third the thermal conductivity was similar to that of the dead brain.

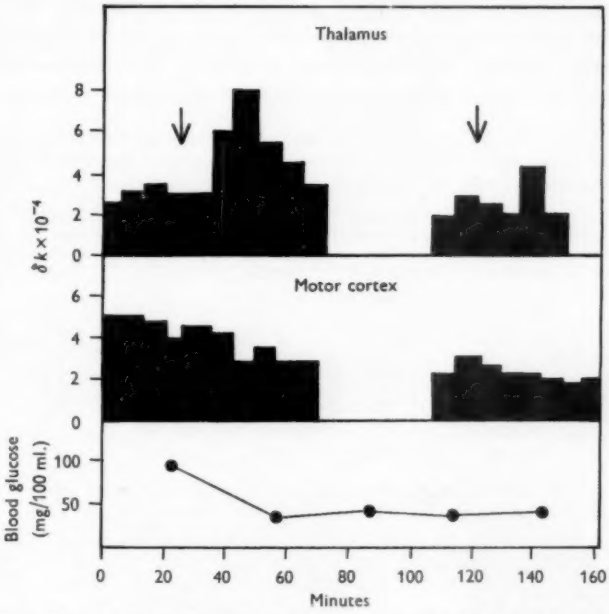

Fig. 2. The effect of insulin (5 i.u./kg) at arrows on blood flow in the thalamus and cerebral motor cortex of the monkey.

The effect of insulin on brain blood flow and metabolic heat production

The effect of insulin on brain blood flow and metabolic heat production was investigated in four monkeys. In some of the experiments recordings were made from all four regions under observation. In such experiments an initial period of recording of 20–30 min was allowed from two regions, during which time steady blood-flow conditions were obtained. Recording was then continued for 20–30 min from the other two recorders. Insulin 5 i.u./kg (Insulin A.B.; British Drug Houses) was then given intravenously and the recording continued for 40 min. Further records were then obtained from the first two recorders.

A second injection of insulin (2·5 i.u./kg) was given 60–70 min after the first, in order to maintain hypoglycaemia. Samples of blood from an indwelling cannula in the femoral artery gave blood-sugar values of from 15 to 43 mg/100 ml., 30–40 min after the first intravenous injection in insulin.

The effect of insulin on brain blood flow. The results of a typical experiment are shown in Fig. 2. The results of observations from the cortical areas of four monkeys are given in Table 2, and from the thalamus in Table 3. It will be seen from Table 2 that the pattern of blood-flow responses from the cortex was variable, either increases or falls in flow being recorded. In the thalamus a more consistent pattern of blood-flow response was

TABLE 2. The effect of insulin (5 i.u./kg) on blood flow (conductivity increment δk) in various parts of the cerebral cortex. The values in brackets were all recorded 40 min after the particular injection referred to. All other values are maximum effects

Expt. no.	Area of cortex	Resting average max. $\delta k \times 10^{-4}$	Average max. δk after 1st insulin	Average max. δk before 2nd insulin	Average max. δk after 2nd insulin
1	Motor	3·0	(1·6)	2·1	2·0
	Sensory	4·4	(4·7)	2·4	2·6
	Occipital	3·7	3·2	2·6	(2·8)
2	Motor	4·8	4·2	2·0	1·7
	Sensory	0·7	(1·7)	1·0	(1·2)
	Occipital	1·3	(1·1)	1·3	(1·3)
3	Sensory	1·7	2·4	1·2	1·2
4	Sensory	2·2	3·9	3·7	3·9

Table 3. The effect of insulin (5 i.u./kg) on blood flow in thalamus (value in brackets = reading after 40 min. All other values = maximum effects)

Expt. no.	Resting average max. $\delta k \times 10^{-4}$	Average max. δk after 1st insulin	Average max. δk before 2nd insulin	Average max. δk after 2nd insulin
1	1·0	2·0	1·1	(1·1)
2	3·3	8·1	2·8	4·1
3	1·5	2·3	1·8	2·4
4	3·6	5·2	2·2	4·2

obtained (Table 3). In the experiment shown in Fig. 2, for example, the injection of insulin was followed by a rise in blood flow to a peak reached within 20 min of the injection. Thereafter, blood flow returned slowly to initial levels despite the maintained hypoglycaemia. A further injection of insulin again produced a significant increase in blood flow without any further change occurring in blood-glucose levels.

The effect of insulin on metabolic heat production. Observations from the cortex in four monkeys are given in Table 4. The immediate responses of the cortical areas to insulin were slight and variable. The most frequent response was a rise in heat production with a maximum within 40 min after the injection. With prolonged hypoglycaemia, however, every obser-

vation except one showed a marked depression of heat production occurring in all the cortical areas examined (Fig. 3). This depression occurred without further change in blood flow and without further change in blood glucose.

TABLE 4. Effect of insulin (5 i.u./kg) on heat production in various parts of the cerebral cortex. Values in brackets were obtained 40 min after insulin

Expt. no.	Area of cortex	Maximal changes in excess temperature ($°C \times 10^{-2}$) after 1st insulin	Maximal changes in excess temperature ($°C \times 10^{-2}$) after 2nd insulin
1	Motor	(−10)	−50
	Sensory	(−16)	−120
	Occipital	−22	(−180)
2	Motor	+18	−50
	Sensory	(+10)	(+16)
	Occipital	(+15)	(−240)
3	Sensory	+16	−185
4	Sensory	+10	−90

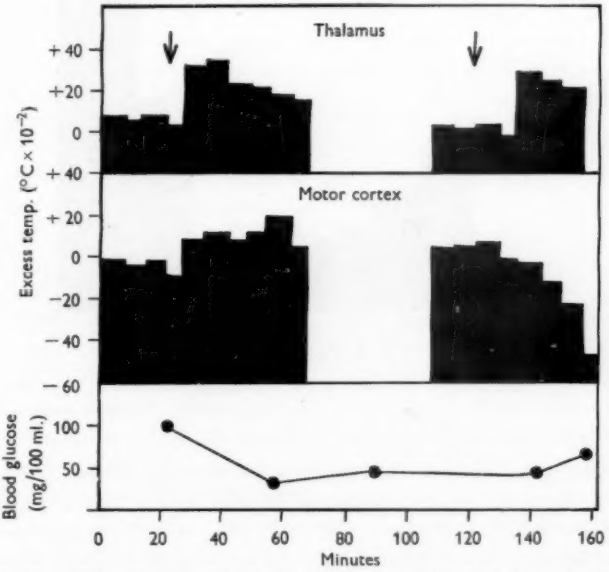

Fig. 3. The effect of insulin (5 i.u./kg) at arrows on metabolic heat production in the thalamus and cerebral cortex of the monkey (same experiment as Fig. 2.)

The results of four experiments on the thalamus are given in Table 5. In all experiments the immediate response to insulin injection was a rise in metabolic heat production accompanying the blood-flow rise already reported. The rise was not maintained and excess temperature slowly fell

towards its initial levels. A second injection also produced a rise in thalamic heat production accompanying the blood-flow change, even though no further change occurred in blood-glucose levels (Fig. 3). In only one experiment was the second injection of insulin followed by a fall in thalamic heat production.

TABLE 5. The effect of insulin (5 i.u./kg) on heat production in the thalamus. The value in brackets was obtained 40 min after insulin

Expt. no.	Maximal change in excess temperature (°C × 10^{-2}) after 1st insulin	Maximal change in excess temperature (°C × 10^{-2}) after 2nd insulin
1	+ 6	(+90)
2	+28	+30
3	+48	+10
4	+18	−30

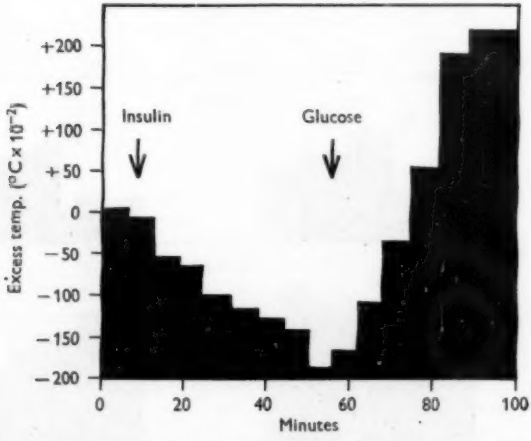

Fig. 4. Depression of heat production in cerebral sensory cortex, produced by a 2nd injection of insulin. Depression reversed by injection of glucose (actual record of later part of experiment given in Fig. 5).

The influence of glucose during prolonged hypoglycaemia. A consistent feature of these results is illustrated in Fig. 4. In this experiment recording was from the sensory cortex. A very considerable depression in heat production occurred without any change in blood-flow or blood-glucose levels after the second injection of insulin. An injection of glucose (1 g/kg) sufficient to restore the levels of blood glucose to 300 mg/100 ml. produced a rapid recovery of heat production, so that calculated excess temperature rose to levels considerably higher than the resting levels. The actual record from this experiment is shown in Fig. 5 to illustrate the magnitude of the response. In three other experiments, glucose, given after a dose of insulin

sufficient to produce cortical depression, always caused recovery, up to or above the resting level.

The effect of glucose on brain blood flow and metabolic heat production

The effect of glucose on brain blood flow and excess temperature was determined in four experiments on different monkeys. After an initial period of recording (30 min) to allow steady blood-flow conditions to be obtained, glucose (1 g/kg) was given intravenously and recording continued for a further 60 min.

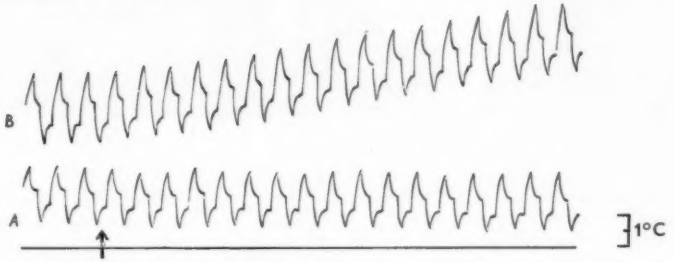

Fig. 5. Photographic trace of the part of the experiment shown in Fig. 4: the effect of glucose on the brain of a hypoglycaemic monkey. *A*, recording from thalamus; *B*, recording from cerebral sensory cortex. Glucose (1 g/kg) given at arrow.

The effect of glucose on brain blood flow. The results of four experiments where blood flow was simultaneously recorded from the thalamus and cerebral cortex are shown in Table 6. A typical result is illustrated in Fig. 6. In the cortex, the effect on blood flow of intravenous glucose, in doses sufficient to raise the blood-glucose level to 300 mg/100 ml. or more, was usually slight. Recordings from the various cortical sites chosen, namely sensory, motor and occipital cortex, showed no difference in reaction to glucose. In only one instance (Expt. 7, Table 6) did any significant rise in blood flow occur.

In the thalamus (Table 6) two of the experiments showed an increase in blood flow. In the other two experiments the flow increases which were recorded were probably not significant.

The effect of glucose on metabolic heat production. The results of four experiments (the same four as are referred to in Table 6) are given in Table 7. A typical result is illustrated in Fig. 6. The findings from the various cortical areas showed slight drops in heat production in three animals, and a marked drop in cortical heat production in another animal.

In the thalamus the results were consistent. Every experiment showed a marked fall in heat production, accompanying the rise in blood flow. The effect was rapid and recovery slow (Fig. 6).

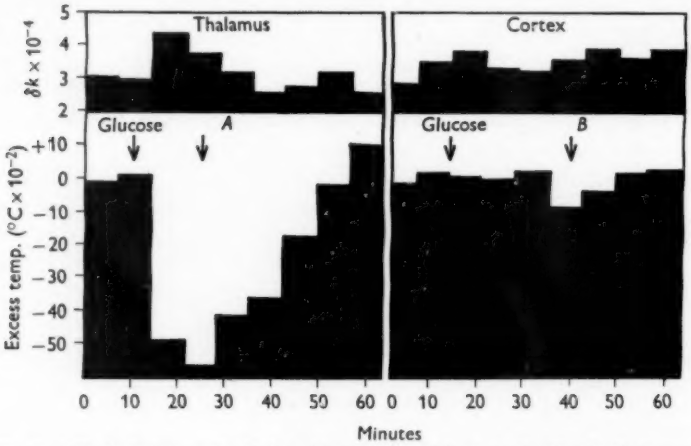

Fig. 6. Effect of glucose (1 g/kg) on blood flow and heat production in thalamus and cortex. Experiments on different monkeys. Blood glucose at A = 300 mg/100 ml. Blood glucose at B = 360 mg/100 ml.

TABLE 6. The effect of glucose (1 g/kg) on blood flow (conductivity increment) in the thalamus and cortex (cortical areas not specified)

	Thalamus		Cortex	
Expt. no.	Resting average max. $\delta k \times 10^{-4}$	Average max. $\delta k \times 10^{-4}$ after glucose	Resting average max. $\delta k \times 10^{-4}$	Average max. $\delta k \times 10^{-4}$ after glucose
5	2·9	4·3	0·6	0·9
6	5·1	5·4	2·7	2·4
7	2·1	2·3	2·3	3·8
8	1·0	2·8	3·5	3·9

TABLE 7. The effect of glucose (1 g/kg) on heat production in the thalamus and cortex

	Max. changes in excess temperature (°C × 10⁻²)	
Expt. no.	Thalamus	Cortex
5	−56	− 4
6	−53	− 6
7	−20	−40
8	−14	− 8

The effect of combined glucose and insulin on brain blood flow and metabolic heat production

In three of the monkeys in which the effect of glucose on brain blood flow and metabolic heat production had been investigated, the effects of

combined glucose (1 g/kg) and insulin (5 i.u./kg) were determined. Intravenous injection was given 3 hr after the previous glucose injection. Blood glucose levels were then between 120 and 180 mg/100 ml.

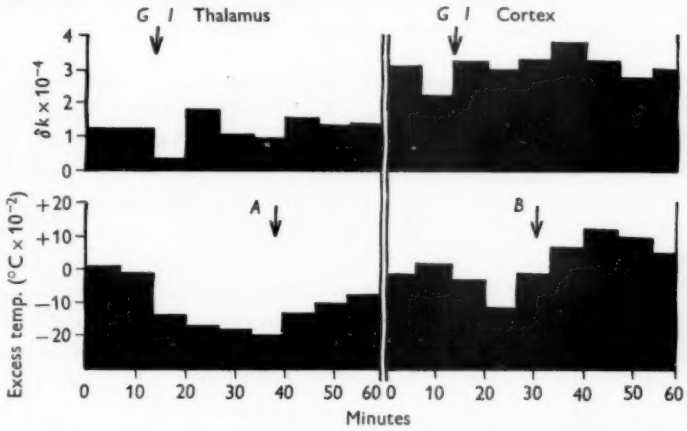

Fig. 7. The effect of glucose (1 g/kg) and insulin (5 i.u./kg) at arrow *GI* on the thalamus and cerebral cortex. Experiments on different monkeys. Blood glucose at A = 330 mg/100 ml. Blood glucose at B = 310 mg/100 ml.

TABLE 8. The effect of combined glucose (1 g/kg) and insulin (5 i.u./kg) on blood flow (conductivity increment) in the thalamus and cortex

	Thalamus		Cortex	
Expt. no.	Resting max. average δk $\times 10^{-4}$	Average max. $\delta k \times 10^{-4}$ after glucose and insulin	Resting max. average δk $\times 10^{-4}$	Average max. $\delta k \times 10^{-4}$ after glucose and insulin
5	1·8	1·9	3·1	3·9
6	3·6	4·5	1·0	2·8
7	1·3	1·8	2·7	2·9

The effect of glucose and insulin on brain blood flow. The results for blood-flow changes in the thalamus and cortex are shown in Table 8, and the pattern of a response in Fig. 7. In each experiment the rise in blood glucose was similar to that obtained in experiments where no insulin was given—namely, to levels higher than 300 mg/100 ml. The effect on blood flow in the various cortical areas and thalamus was variable. In one experiment both the cortex and thalamus showed immediate rises in blood flow. In the other experiments there were either smaller increases or no change in blood flow.

The effect of glucose and insulin on heat production. Metabolic heat production was calculated in the same experiments recorded in Table 8 and the results are shown in Table 9 and Fig. 7. In the cortical areas there was

an initial depression of varying extent, always short-lived and usually followed by a rise above initial levels. In the thalamus the results were similar to those reported above where glucose alone was given. There was an initial depression of considerable magnitude, quick to develop and only slowly returning towards resting levels.

TABLE 9. The effect of combined glucose (1 g/kg) and insulin (5 i.u./kg) on heat production in the thalamus and cortex

Expt. no.	Maximum changes in excess temperature ($°C \times 10^{-3}$) Thalamus	Cortex
5	−72	−12
6	−32	− 7
7	−20	−20

DISCUSSION

Internal calorimetry is a technique which enables both blood flow and metabolic changes to be evaluated from the area in which the recorder is inserted. The use of excess temperature as an indicator of heat production has been justified with respect to liver and muscle. (Dosekun *et al.* 1960). It is equally valid in brain. It is a measure of temperature change in a heated thermocouple after allowance has been made for the temperature changes produced by blood flow. Provided the point of reference, the cold junction, is in the blood stream supplying the organ, and the environment is at a constant temperature and heat losses are constant, it is contended that the only factor, other than blood flow, which can affect the heated thermocouple is local metabolic heat production.

Resting blood-flow distribution in the lightly anaesthetized monkey brain showed wide variations from animal to animal, but the average findings from motor, sensory and occipital cortex indicated little difference in blood-flow distribution between any of these areas or between these areas and the thalamus. Blood flow in the subcortical white matter was low—so low as not to be measurable by this method.

Measurement of metabolic heat production showed no differences in the pattern of reaction between the various cortical areas investigated, but the thalamus was markedly different in its behaviour. In the cortex insulin injections produced first a slight rise in metabolic activity, with little change in blood flow. After prolonged hypoglycaemia there was a marked depression of cortical heat production, a gross change reversible by the giving of glucose.

In the thalamus, however, there was a marked rise in heat production with insulin accompanied by a rise in blood flow. In only one experiment was there any fall in heat production, even after hypoglycaemia prolonged for over an hour.

Glucose injections had rather different effects. The cortex showed little reaction either in blood flow or heat production. The thalamus, however, always responded with a marked depression of heat production, even if insulin was given simultaneously, which may be explained on the grounds that insulin is slow to act, and that there was always an initial rise in blood glucose.

These results are best interpreted as being directly related to levels of blood glucose. They are differences of degree rather than of kind. It would seem that the cortex is more sensitive to low levels of blood glucose, whereas the thalamus is more sensitive to high levels of blood glucose.

The findings are consistent with the suggestion that the thalamus houses some reserves of glucose, making it less sensitive to low blood-glucose levels than the cortex; that even in the cortex, since the final depression is a late event, there must also be some glucose storage capacity. Under these circumstances it might be reasonable to suppose that release of glucose, from whatever substrate may be involved, is an exothermic process, and that the final cortical depression represents a state of exhaustion of those stores.

The thalamic depression in the presence of high blood-glucose levels is more difficult to explain. If one assumes release of glucose from intra-thalamic stores to be an exothermic process, and if one assumes that continued release of such glucose is a source of continuous heat production, then in the presence of high blood glucose it would not be unreasonable to presume that glucose release would be slowed down. On the basis of the above assumptions this might well produce a fall in thalamic heat production of the kind observed.

These speculations are merely put forward in an attempt to delineate the further problem. The present technique may be regarded as a biochemical indicator which amongst other things demonstrates where the biochemical problems lie. Clearly there is a strong case to be made for the reassessment of the basic biochemistry of these parts of the brain. Whether the small amounts of glycogen reported in the cortex (Kerr & Ghantus, 1936) can account for the phenomena reported or whether another substrate is involved is open to question. Little tissue-slice work seems to have been done on the thalamus and there is a need for the further biochemical investigation of this organ in relation to the cortex.

SUMMARY

1. The technique of 'internal calorimetry' was applied to the investigation of blood flow and heat production in the sensory, motor and occipital areas of the cerebral cortex and in the thalamus of West African Green monkeys.

2. Insulin produced a small rise in heat production in all cortical areas and a much bigger rise in heat production in the thalamus. Protracted hypoglycaemia brought about a gross fall in cortical heat, reversible by the administration of glucose. The thalamus was much more resistant and only rarely did gross hypoglycaemia cause a fall in its heat production.

3. Insulin was followed by small changes in cortical blood flows but by a marked rise in thalamic blood flow.

4. Glucose alone had little effect either on blood flows or on heat production in the cerebral cortex. In the thalamus, however, glucose administration always caused a marked drop in heat production.

5. Possible interpretations of these effects are discussed.

The authors are indebted to the West African Council for Medical Research, the Medical Research Council of Great Britain and the Stewart Haley Trust for their assistance.

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THE ABSORPTION OF SUCROSE, MALTOSE AND HIGHER OLIGOSACCHARIDES FROM THE ISOLATED RAT SMALL INTESTINE

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Several workers have studied the absorption of sugars from preparations *in vitro* of the small intestine of various species (e.g. Fisher & Parsons, 1950; Darlington & Quastel, 1953; Wilson & Vincent, 1955). In general, quantitative results have been obtained only with monosaccharides because of the limitations of the analytical methods. The availability of an automatic scanning method for paper chromatography of radioactive substances (Chain, Frank, Pocchiari, Rossi, Ugolini & Ugolini, 1956) has made it possible to examine quantitatively the absorption of glucose, maltose and the higher oligosaccharides contained in a starch hydrolysate (similar to liquid glucose, B.P. Codex 1959), and of sucrose, from the isolated surviving rat small intestine.

METHODS

Animals. Male Wistar rats weighing 200–250 g and maintained on a standard diet were used.

Radioactive materials. Uniformly labelled ^{14}C-glucose, ^{14}C-sucrose and ^{14}C-fructose were obtained from the Radiochemical Centre, Amersham, Bucks. ^{14}C-labelled tobacco leaf starch was prepared by a modification (P. F. Langley & K. D. Rapson, personal communication) of the method of Porter & Martin (1952). Hydrolysis was carried out with 10 parts (w/v) of 0·1 N-HCl at 145° C for 45 min. This procedure was comparable with that used to produce liquid glucose from maize starch. This product was analysed chromatographically and shown to correspond closely with a typical liquid glucose (Table 1), the data for which were obtained partly from analyses published by the Technical Advisory Bureau, Corn Industries Research Foundation (1956). All sugars were diluted with the corresponding inert material to give a specific activity of about 1·0 μc/mg.

Intestinal preparation. The preparation of the rat small intestine was similar to that described by Fisher & Parsons (1949). An oxygenated (95 % O_2, 5 % CO_2) Krebs–Ringer bicarbonate buffer solution was circulated by gas (95 % O_2, 5 % CO_2) lift through the whole length of the small intestine (from the duodenojejunal flexure to the caecum) through cannulae inserted into both ends of the lumen in an ether-anaesthetized rat. The intestine was then removed from the animal and suspended in a bath containing 50 ml. of Krebs–Ringer solution which was also circulated by gas lift. By this technique the tissue was prevented from being subjected to anoxia at any time, and thus the associated disturbances demonstrated by Fisher & Parsons (1949) were avoided.

Perfusion conditions. All perfusions were carried out for 1 hr at 37° C with an initial volume of 50 ml. of Krebs–Ringer bicarbonate buffer at pH 7·2 containing an initial concentration of 0·5 % (w/v) of each sugar, except where otherwise stated. 1 ml. of 0·3 % phenol red/50 ml. of buffer was added to the inner circuit to reveal any minute pinholes in the neighbourhood of the cannulae. As soon as the circulation was set up, a 1 ml. sample was removed and analysed for total radioactivity and the initial circulating volume thus calculated to correct for saline entrapped in the lumen during the operation procedure. The course of the absorption of the different sugars was followed by taking 1 ml. samples from the inner and outer circuits at intervals during the next 60 min. These were immediately replaced by 1 ml. of Krebs–Ringer buffer to prevent volume changes, appropriate corrections for this sampling being made in the calculations.

TABLE 1

	Anhydrous sugars (%)	
	Hydrolysed tobacco starch	Liquid glucose from maize starch
Glucose	16·2	18·9
Maltose	11·4	14·0
Maltotriose	10·0	11·0
Maltotetraose	9·4	9·7
Maltopentaose	8·6	8·4
Higher oligosaccharides	44·4	38·0
	100·0	100·0

At the end of the perfusion period the contents of the inner and outer circuits were both drained and both circuits washed with 50 ml. of buffer circulated for 1 min. From the total radioactivity in the washings obtained from the inner and outer circuits a correction was made to allow for the loss in volume due to incomplete drainage of the circuits. A correction for evaporation losses was also applied.

Preparation of samples for chromatography. Paper chromatograms were made of tissue extracts and the samples of inner and outer circulating fluids. The tissue extracts were prepared as follows. The intestine, after measurement of its length, was transferred into 10 ml. of ice-cold 60 % alcohol and homogenized in a Waring Blendor. After homogenization the solution was centrifuged and the supernatant collected. The centrifuged tissue was again homogenized in 10 ml. alcohol (this time in a Potter–Elvehjem homogenizer) and centrifuged; and this procedure was repeated three times. The combined extracts were then evaporated to dryness in a Craig rotary evaporator. The extract was then dissolved in 1 ml. of water and samples removed for paper chromatography. The alcohol-insoluble material was kept for determination of residual radioactivity.

The samples of inner and outer fluids removed during and at the end of the 60 min perfusion were freeze-dried and reconstituted in one-fifth of the original volume, in order to provide sufficient radioactivity for precise counting on paper chromatograms.

Paper chromatography. The concentrated samples were analysed by both uni- and bi-dimensional paper chromatography. Each sample was chromatographed in at least two solvent systems at 20° C. The following solvents were used:

A. *n*-butanol:ethanol:water–2:1:1.
B. *n*-butanol:acetic acid:water–40:11:25.
C. *sec.*-butanol:formic acid (88 %):water–5:1:1.
D. phenol:ammonia (0·880):water–80:1:20.
E. phenol:acetic acid:water–80:1:20.

For the separation of the components of starch hydrolysate, unidimensional chromatograms were run for 60 hr in solvent *A*. Bidimensional chromatograms were run first in

solvent *A* for 36 hr and then in solvent *C* for 36 hr. Lactate was determined on unidimensional chromatograms run for 19 hr in solvent *B*. Both lactate and amino acids were determined on bidimensional chromatograms run first in solvent *C* for 16 hr and then in solvent *D* for 19 hr. Glucose, fructose and sucrose were determined on bidimensional chromatograms run either for 36 hr in solvent *A* and then for 36–48 hr in solvent *C*, or for 19 hr in solvent *B* followed by 24 hr in solvent *E*.

In order to obtain clearly defined spots on the chromatograms of samples containing large amounts of sucrose with small amounts of glucose, desalting was carried out with Amberlite MB3 resin in the bicarbonate form (Woolf, 1953).

To prevent losses of lactate by volatilization, all chromatograms used for the separation of lactate were placed in an atmosphere of ammonia vapour immediately after removal from the solvent tank.

Quantitative determination. The paper chromatograms of the radioactive sugars and their metabolites were examined by the automatic scanning device of Chain *et al.* (1956) in its modified form (Frank, Chain, Pocchiari & Rossi, 1959).

The radioactivity of the insoluble residue was determined by counting thin films of the solid material on aluminium planchets. Correction for self-absorption and counter geometry were applied to make the results comparable with measurements of the radioactivity counted on paper.

RESULTS

Rates of absorption

The mean absorptions of the different sugars calculated from the quantitative evaluation of the scanned chromatograms are given in Table 2. At comparable concentration (0·5 % (w/v)) the highest rates of disappearance of total carbohydrate from the lumen and the highest rates of appearance of glucose on the serosal side were obtained when starch hydrolysate or glucose were perfused. The figures given for the experiments with glucose are in close agreement with the published rates of Fisher & Parsons (1953). The rate of disappearance of sucrose was greater than that of fructose and more fructose appeared on the serosal side after perfusion with sucrose

TABLE 2. Absorption of carbohydrates during perfusion of rat small intestine with various sugars

esults expressed as mg carbohydrate/cm (mean ± S.E.) after 1 hr perfusion carried out at 37° C ml. of oxygenated (95 % O_2, 5 % CO_2) Krebs–Ringer bicarbonate buffer at pH 7·2. All sugars were at a concentration of 0·5 % (w/v) except where indicated.

Sugar perfused	No. of expts.	Disappearance of carbohydrate from inner fluid			Appearance of carbohydrate in outer fluid		
		Glucose	Fructose	Total	Glucose	Fructose	Total
h hydrolysate	8	—	—	1·98 ± 0·05	0·96 ± 0·04	nil	0·96 ± 0·04
ose	4	1·81 ± 0·03	—	1·81 ± 0·03	1·18 ± 0·03	nil	1·18 ± 0·03
ose-fructose ture	3	1·22 ± 0·04	0·34 ± 0·03	1·56 ± 0·03	0·57 ± 0·08	0·06 ± 0·02	0·62 ± 0·09
ose-fructose ture*	2	1·77 ± 0·01	0·68 ± 0·02	2·45 ± 0·01	0·98 ± 0·01	0·11 ± 0·01	1·09 ± 0·01
ose	4	—	—	1·15 ± 0·05	0·30 ± 0·02	0·17 ± 0·02	0·47 ± 0·02
tose	3	—	0·79 ± 0·05	0·79 ± 0·05	nil	0·11	0·11

* 1·0 % (w/v) concentration used in these experiments.

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Sugar perfused	No. of expts.	Disappearance of carbohydrate from inner fluid			Appearance of carbohydrate in outer fluid		
		Glucose	Fructose	Total	Glucose	Fructose	Total
tarch hydrolysate	8	—	—	1·98 ± 0·05	0·96 ± 0·04	nil	0·96 ± 0·04
lucose	4	1·81 ± 0·03	—	1·81 ± 0·03	1·18 ± 0·03	nil	1·18 ± 0·03
lucose-fructose mixture	3	1·22 ± 0·04	0·34 ± 0·03	1·56 ± 0·03	0·57 ± 0·08	0·06 ± 0·02	0·62 ± 0·09
lucose-fructose mixture*	2	1·77 ± 0·01	0·68 ± 0·02	2·45 ± 0·01	0·98 ± 0·01	0·11 ± 0·01	1·09 ± 0·01
ucrose	4	—	—	1·15 ± 0·05	0·30 ± 0·02	0·17 ± 0·02	0·47 ± 0·02
ructose	3	—	0·79 ± 0·05	0·79 ± 0·05	nil	0·11	0·11

* 1·0 % (w/v) concentration used in these experiments.

(a)
1
2
3
4
5
6

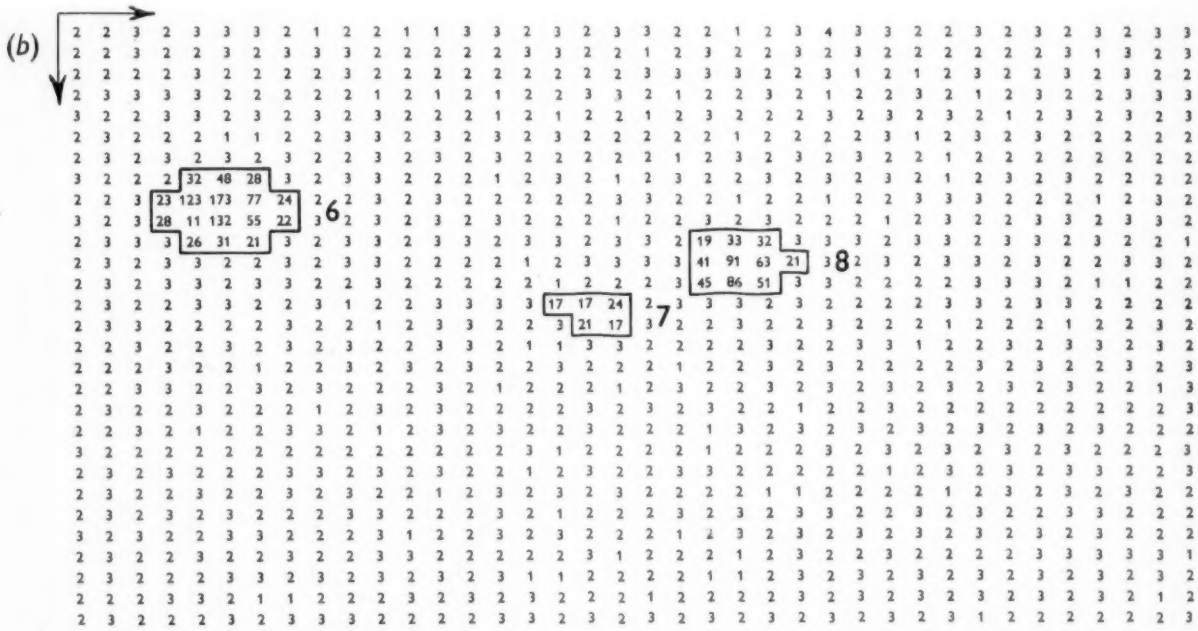

Fig. 1. Number maps of radiochromatograms of: (*a*) starch hydrolysate remaining in the inner fluid after perfusion for 1 hr; (*b*) serosal fluid after perfusion with starch hydrolysate. Chromatogram (*a*) developed in → solvent *A*, ↓ solvent *C*; chromatogram (*b*) in → solvent *C*, ↓ solvent *D*. (1) polysaccharides, (2) maltopentaose, (3) maltotetraose, (4) maltotriose, (5) maltose, (6) glucose, (7) alanine, (8) lactate.

than after perfusion with fructose. The rate of disappearance of fructose was about 45 % of the rate observed with glucose, in good agreement with the results of Cori (1925) and Verzár & McDougall (1936) with *in vivo* techniques. With a mixture of glucose and fructose, each in a concentration of 0·5 % (w/v), the rates of absorption were not significantly different from the rates observed with each of the sugars perfused alone.

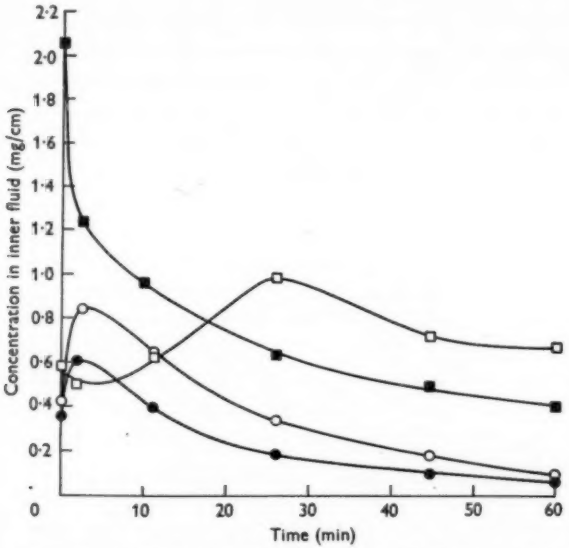

Fig. 2. Composition of the inner fluid at various periods during perfusion of the rat small intestine with starch hydrolysate; each point represents the mean of two experiments. □ Glucose, ◐ maltose, ○ maltotriose, ■ maltotetraose + polysaccharides.

Starch hydrolysate. The composition of the inner fluid during perfusion with starch hydrolysate is shown in Fig. 3, and an autoscanner number map of a chromatogram of the inner fluid at the end of the perfusion is shown in Fig. 1(*a*). The concentration of the higher oligosaccharides decreased rapidly while the maltose concentration rose steeply, indicating strong amylase activity in the lumen (Fig. 2). Following the steep rise, the maltose concentration diminished and during the first 30 min the glucose concentration increased, indicating maltase activity. Of the sugars in starch hydrolysate, only glucose appeared on the serosal side (see Fig. 1(*b*)).

The time course of the absorption of starch hydrolysate from the lumen and the appearance of glucose on the serosal side is given in Table 3. The rate of disappearance of starch hydrolysate from the inner fluid during the

first half hour was 2·7 mg/cm/hr, whereas in the second half hour it dropped to 1·54 mg/cm/hr. The corresponding rates of appearance on the serosal side were 0·68 mg/cm/hr in the first half hour and 1·32 mg/cm/hr in the second half hour. Although the concentration of free glucose in the lumen was never higher than 0·16 % (w/v), the rate of appearance of glucose on the serosal side during perfusion with starch hydrolysate was as high as the mean rate obtained with 0·5 % (w/v) glucose in the lumen.

TABLE 3. Rate of absorption of carbohydrates during perfusion of rat small intestine with starch hydrolysate

Results expressed as mg carbohydrate/cm intestine/hr (mean of 4 expts.) during perfusion carried out with 0·5 % (w/v) starch hydrolysate.

Time (min)	Rate of disappearance of carbohydrate from lumen	Rate of appearance of glucose in serosal fluid
0–10	3·00	0·60
10–20	2·76	0·60
20–30	2·34	0·84
30–40	1·92	1·32
40–50	1·56	1·32
50–60	1·14	1·32

Sucrose. The composition of the inner circuit during perfusion with sucrose is shown in Fig. 3. Sucrose was not completely hydrolysed during the perfusion. The concentration of glucose in the lumen was small and tended to fall during the second 30 min of perfusion, as its rate of absorption was greater than its rate of formation from sucrose. The concentration of fructose in the lumen rose during the perfusion because of its lower rate of absorption.

The appearance of glucose and fructose in the serosal circuit during perfusion with sucrose is compared in Fig. 4 with the glucose appearing after perfusion with starch hydrolysate. With starch hydrolysate the concentration of glucose on the serosal side was approximately twice the sum of the glucose and fructose concentrations from sucrose. With a 1:1 glucose-fructose mixture (total concentration 0·5 % (w/v)) the amount of sugar appearing on the serosal side was still only half that obtained with 0·5% (w/v) starch hydrolysate.

Fructose was found to be absorbed in the rat small intestine without epimerization to glucose. In the guinea-pig, however, 36 % of the carbohydrate appearing on the serosal side after the perfusion was found to be glucose (Table 4).

Because fructose was removed from the lumen at a slower rate than glucose, the proportion of fructose to glucose in the lumen rose steeply during the perfusions with glucose-fructose mixture (1:1). At the end of the 1 hr perfusion the carbohydrate in the lumen consisted of 84–97 %

fructose whereas the serosal fluid carbohydrate consisted of 89–93% glucose.

Metabolism of sugars and transport of water. With all the sugars studied some conversion to lactate was observed. Lactate was found in both the inner and outer circuits and in the extract of the intestinal tissue. The

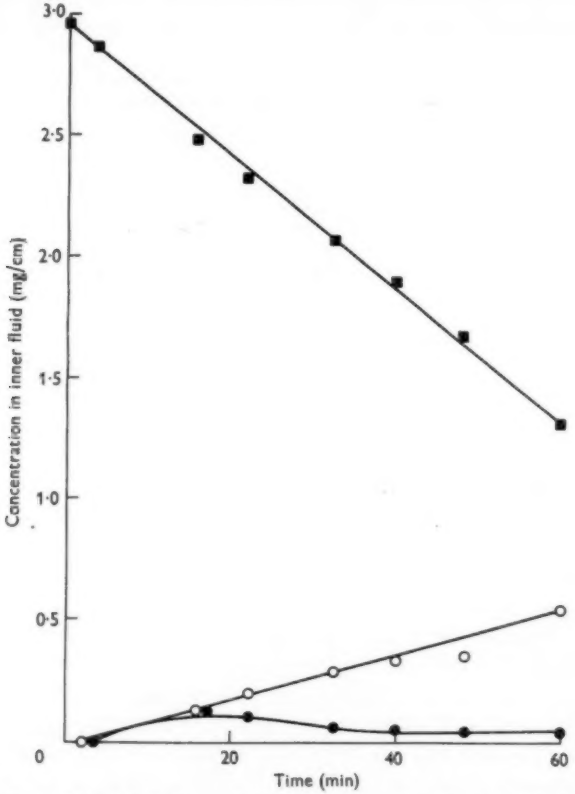

Fig. 3. Composition of the inner fluid at various periods during perfusion with sucrose; each point represents the mean of four experiments. ■ Sucrose, ● glucose, ○ fructose.

distribution of this lactate and the total determined is shown in Table 5. With all the sugars examined alanine was detected in trace amounts in the intestinal extracts and low residual radioactivity was associated with the insoluble intestinal residue.

The observation of Fisher & Parsons (1950), that during absorption of glucose there was considerable water transport against a large osmotic

pressure gradient was confirmed. In the present experiments a loss of approximately 150 μl./cm of intestine was observed when starch hydrolysate or glucose was perfused. This was reduced to about 100 μl./cm with sucrose and to 50–60 μl./cm with fructose perfusions.

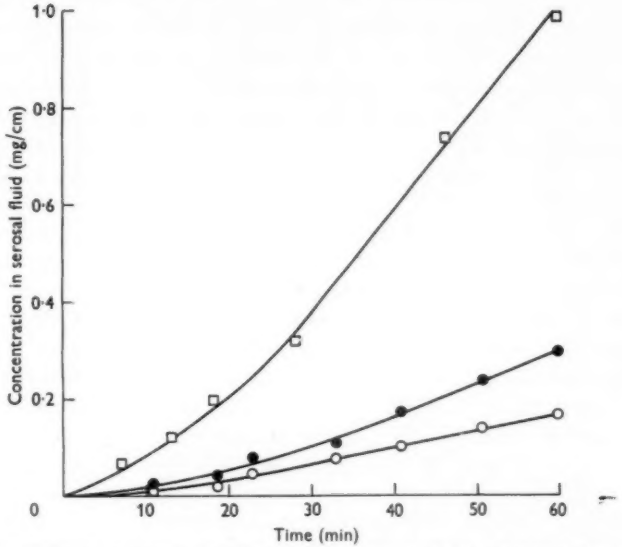

Fig. 4. Appearance of sugars at various times in the serosal fluid after perfusion of the rat small intestine with starch hydrolysate or sucrose; each point represents the mean of four experiments. □ Glucose after perfusion with starch hydrolysate, ■ glucose after perfusion with sucrose, ○ fructose after perfusion with sucrose.

TABLE 4. Absorption of fructose during perfusion of guinea-pig small intestine

Results expressed as mg sugar/cm intestine after perfusion for 1 hr with 0·5 % (w/v) fructose.

Disappearance of fructose from lumen	Appearance of sugar on serosal side		
	Fructose	Glucose	Total
0·91	0·14	0·08	0·22

TABLE 5. Formation and distribution of lactate after perfusion with various sugars

Results expressed as sugar converted to lactate, mg/cm/hr. Experimental conditions as given in Table 2.

	Inner circuit	Outer circuit	Intestinal extract	Total ± S.E.
Starch hydrolysate	0·091	0·331	0·088	0·510 ± 0·052
Sucrose	0·064	0·205	0·063	0·332 ± 0·041
Fructose	0·055	0·051	0·057	0·163 ± 6·022
Glucose	0·086	0·178	0·085	0·349 ± 0·033
Glucose-fructose 1:1	0·094	0·224	0·098	0·416 ± 0·052

DISCUSSION

The rate of disappearance of carbohydrate from the lumen of the isolated rat small intestine was certainly not less when starch hydrolysate was used than when 0·5 % (w/v) glucose was used, although with starch hydrolysate the concentration of glucose in the lumen never rose above 0·16 % (w/v). It is of interest to compare the observed rates of disappearance from the lumen and appearance on the serosal side with the results of Fisher & Parsons (1953) using various glucose concentrations in the inner circuit. With a 0·16% (w/v) glucose concentration in the lumen, the rate of disappearance from the lumen of glucose would be expected to be around 1 mg/cm/hr. As this 1 mg/cm/hr of glucose corresponds to the glucose utilization rate for the intestine (Fisher & Parsons, 1953) practically no glucose would be expected on the serosal side under these conditions. The appearance of 1·32 mg/cm/hr (Table 2) of glucose on the serosal side in the second half hour of the starch hydrolysate perfusions, when the glucose concentration was at most 0·16 % (w/v), cannot therefore be accounted for solely by glucose absorption. Something other than glucose must be absorbed from the lumen and this substance must be supplying the needs of the intestinal wall for glucose.

The time course of the absorption of starch hydrolysate from the lumen was markedly different from the published results of Fisher & Parsons (1953) with glucose. The rate of glucose absorption from a solution containing initially 0·5 % (w/v) was 2·54 mg/cm/hr in the first 30 min but only 1·18 mg/cm/hr in the second 30 min (Fisher & Parsons, 1953). With starch hydrolysate the corresponding rates were 2·70 mg/cm/hr and 1·54 mg/cm/hr (Table 3). The rates of appearance of glucose in the serosal fluid with perfused glucose (Fisher & Parsons, 1953) were 1·16 mg/cm/hr for the first 30 min and only 0·10 mg/cm/hr for the last 30 min. With starch hydrolysate the corresponding rates were 0·68 mg/cm/hr and 1·32 mg/cm/hr (Table 3). With starch hydrolysate, therefore, an apparent time lag was observed before the maximum rate of appearance of glucose in the serosal fluid was attained.

From these differences in the results of glucose and starch hydrolysate perfusions there is definite evidence for the direct absorption of maltose, maltotriose or the higher oligosaccharides of starch hydrolysate from the lumen of the rat small intestine. When the comparisons between glucose and starch hydrolysate are made in terms of the glucose equivalent, the initial concentration of starch hydrolysate becomes 0·53 % (w/v). On average the rates of disappearance of starch hydrolysate from the lumen are 8–9 % greater when thus expressed than when expressed as weights of total carbohydrate. The mean rate of disappearance of 0·53 % (w/v) starch

hydrolysate then becomes $2{\cdot}15 \pm 0{\cdot}05$ mg/cm/hr, which is 19 % greater in terms of glucose equivalent than the rate of disappearance from 0·5 % (w/v) glucose (Table 1). From the kind of relation known to exist between glucose concentration and rate of absorption (Fisher & Parsons, 1953) the 19 % difference in rate could not result solely from the 6 % difference in concentrations. The differences described above between the rates of absorption of starch hydrolysate and glucose in the first and second half hours are similarly accentuated by conversion to glucose equivalent.

Additional evidence for the absorption of higher saccharides was provided by the results of the perfusions with sucrose. Sucrose, although only partially inverted during the perfusion, was removed from the lumen faster than fructose, and the rate of appearance of fructose on the serosal side was higher with sucrose in the lumen than with fructose alone. Sucrose must therefore be absorbed as such by the mucosa with subsequent breakdown to glucose and fructose in the intestinal wall. Somewhat similar evidence for the absorption of sucrose in the guinea-pig was presented by Fridhandler & Quastel (1955). These authors, using much higher sucrose concentrations than in the present experiments, were able to demonstrate the presence of unchanged sucrose on the serosal side. In the present experiments radioactive sucrose was not found on the serosal side and only traces were detected in the alcoholic extract of the intestine.

Fructose absorption follows different pathways in the rat and guinea-pig. In the former species fructose was absorbed at a higher rate than the published rate for sorbose (Cori, 1925; Darlington & Quastel, 1953), which was considered to be absorbed by passive diffusion. In the guinea-pig partial epimerization to glucose was observed, confirming the results of Darlington & Quastel (1953). Ginsberg & Hers (1959) have put forward a hypothesis for this species difference, based on the presence or absence of glucose-6-phosphatase activity in the mucosa. It is of interest that some water transport was observed across the intestinal wall during fructose absorption. Although the rate of water transport was much lower here than during glucose perfusion, it is evident that this water transport is not specifically linked with glucose absorption in the rat small intestine.

Only trace amounts of alanine and insoluble material were formed from the sugars, and the lactate produced by utilization of the sugars during absorption formed only 15–25 % of the total sugar disappearing from the lumen. The distribution of this lactate was found to vary according to the amount of water transport associated with the absorptive process.

SUMMARY

1. The absorption from the isolated rat small intestine of sucrose, a starch hydrolysate similar to liquid glucose B.P. Codex 1959, glucose and

fructose was studied by means of a quantitative radioactive paper-chromatographic technique.

2. From the observed rates of absorption of starch hydrolysate and the published results for glucose absorption it was concluded that something other than glucose was absorbed during perfusion with starch hydrolysate, and that this substance (maltose, maltotriose or a higher saccharide) was able to supply the needs of the intestinal wall for glucose.

3. Only glucose appeared on the serosal side after starch hydrolysate perfusion. Sucrose was partially inverted during absorption and both glucose and fructose appeared on the serosal side.

4. The perfusions with sucrose led to a higher concentration of fructose on the serosal side than after perfusions with fructose. It was concluded that sucrose was being absorbed as such by the mucosa, with subsequent break-down to glucose and fructose in the intestinal wall.

5. The rates of disappearance from the lumen of starch hydrolysate and glucose were about twice those of sucrose and fructose. The rate of appearance of sugar on the serosal side after perfusion with starch hydrolysate or glucose was twice that of sucrose and nearly ten times that of fructose.

6. No conversion of fructose to glucose was observed in perfusion of the rat small intestine with fructose, whereas in the guinea-pig considerable conversion occurred.

7. All sugars examined were converted to lactate during the absorption process to the extent of 15–25% of the total sugar disappearing from the lumen. Traces of alanine were found in the intestinal wall together with small amounts of radioactive insoluble material.

The authors are indebted to Professor R. B. Fisher for helpful discussions and to Mr D. F. Broadbridge for advice on the preparation of tobacco leaf starch hydrolysate similar to liquid glucose B.P. Codex 1959. They gratefully acknowledge the skilled technical assistance of Mrs Brenda Mansford and the work of Mr P. F. Langley and Dr H. D. C. Rapson of Beecham Research Laboratories Ltd. on the preparation of the ^{14}C-tobacco leaf starch.

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ON THE PERMEABILITY OF END-PLATE MEMBRANE DURING THE ACTION OF TRANSMITTER

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Movements of specific ions in excitable tissues have been emphasized by a number of recent experiments. In particular, there have been many quantitative experiments which show that the rising phase of the nerve impulse in invertebrate giant axons is associated with an inflow of sodium and the falling phase with an outflow of potassium (Hodgkin, Huxley & Katz, 1952; Hodgkin & Huxley, 1952) and similar selective permeability changes have been observed accompanying the propagated action potential in muscle fibres and in myelinated nerve fibres (Dodge & Frankenhaeuser, 1959; Hodgkin & Horowicz, 1959*a*).

In addition, the inhibitory potentials observed in some nerve cells (Coombs, Eccles & Fatt, 1955; Edwards & Hagiwara, 1959), heart muscle fibres (Burgen & Terroux, 1953; Hutter & Trautwein, 1956; Trautwein & Dudel, 1958), and crustacean muscle (Fatt & Katz, 1953; Boistel & Fatt, 1958) have been shown to be produced by changes in permeability of the post-synaptic membrane to potassium and/or chloride ions.

On the other hand, it has been considered that at the end-plate the transmitter produces a rapid simultaneous transfer of sodium and potassium, and possibly also of all other free ions on either side of the membrane (Fatt & Katz, 1951; del Castillo & Katz, 1954, 1955, 1956). An approximately linear relationship has been observed between the amplitude of the end-plate potential (e.p.p.) and the membrane potential, the equilibrium potential being about -15 mV (Fatt & Katz, 1951). A similar relationship has also been observed in the amplitude of end-plate current (e.p.c.) obtained when the membrane was clamped at a constant potential during neuromuscular transmission (Takeuchi & Takeuchi, 1959). The membrane potential at which e.p.c. becomes zero may be called provisionally 'e.p.c. equilibrium potential'. In the present experiment, the e.p.c. equilibrium potential was measured in solutions of various ionic composition and the ions which contributed to the e.p.c. were determined. It will be shown

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that during the action of transmitter the end-plate becomes permeable mainly to sodium and potassium ions, but probably not to chloride ions.

METHODS

The sartorius muscle was dissected with the sciatic nerve from winter frogs (*Rana pipiens*) and mounted in a chamber made of methacrylate resin (lucite). The nerve was introduced into a wet chamber and stimuli were applied by a pair of silver electrodes. The capacity of the muscle chamber was about 4 ml. When the ionic composition was changed, at least 20 ml. of solution was exchanged by a different solution and the preparation was usually left for 10 min in each solution before measurements were made, except in chloride-deficient solution. When potassium-deficient solution was used, the fluid was kept flowing during the experiment.

The voltage clamp technique was similar to that previously reported (Takeuchi & Takeuchi, 1959), although a slightly modified amplifier for current recording was used. In order to change the clamped membrane potential, a square voltage pulse of about 15 msec duration was applied to the middle stage of the negative feed-back amplifier. A relatively short pulse for changing membrane potential has the advantage of avoiding a possible change in potassium concentration in nearby muscle fibres, which would occur if long pulses or d.c. had been used (Takeuchi & Takeuchi, unpublished). However, the short pulse has the disadvantage that when electrodes were inserted somewhat away from the end-plate, the measured e.p.c. equilibrium potential would tend to assume a lower value than that obtained with longer pulses.

Intracellular electrodes filled with 3 M-KCl were used for recording potential changes and, in most cases, for passing current. In some cases, especially when chloride concentration was changed, 0·6 M potassium-sulphate- or 1·5 M sodium-citrate-filled electrodes were used for current electrodes. In experiments employing electrophoretic injection of sodium ions into muscle fibres, electrodes filled with 3 M-NaCl or 1·5 M sodium citrate were used for passing current.

Electrodes were inserted under microscopic control and the end-plate was located exactly by inserting the electrode at several points along the length of muscle fibre. In order to ensure accurate localization of the electrodes at the end-plate, two criteria were used: (1) the e.p.p. had to show maximal amplitude and shortest rise time at the site of electrode impalement, and (2) the e.p.c.–membrane-potential relationship had to intercept the voltage axis at -10 to -20 mV. If the electrodes were slightly away from an end-plate focus, the measured e.p.c. equilibrium potential tended to approach zero. Generally the current-passing electrode was left in the fibre during the entire experimental series in order to maintain this localization. The recording electrode could then be withdrawn during changing of solutions.

The membrane potential of muscle may be defined as the difference between potentials recorded at points just outside the muscle membrane and inside the membrane. The tip potential may change in different ionic composition, and this might influence the value of the resting potentials measured in various solutions. Relatively low-resistance KCl-filled electrodes were used for potential recording and this served to minimize the change in tip potential in different media (Adrian, 1956). Before and after each series of experiments the recording electrode was withdrawn and the resting potential and the drift of recording system checked. The resting potential tended usually to decrease with time, but during the experiment the membrane potential was clamped within ± 1 mV of the previously determined value.

The normal Ringer's solution used had the following ionic composition (mM): Na^+ 113·6; K^+ 2·5; Ca^{2+} 2·0; Cl^- 117·5; HPO_4^{2-} 1·1; $H_2PO_4^-$ 0·4. In addition, tubocurarine chloride (3×10^{-6} g/ml.) was added to block neuromuscular transmission. Sodium-deficient solutions were obtained by mixing normal Ringer's solution and isotonic sucrose or dextrose solution

containing the same concentrations of K^+, Ca^{2+} and phosphate buffer as normal solution. Sodium-rich solutions were made by adding crystalline NaCl. In lower concentrations of sodium (less than about 35 mM) neuromuscular transmission was blocked, so that tubocurarine was omitted. Solutions with sodium concentration less than 20 mM were not used, because at room temperature conduction block of the nerve frequently occurred in these solutions.

The concentration of potassium ions was altered by replacing sodium chloride by potassium sulphate, concentrations of other ions being kept constant. When the concentration of potassium ion in Ringer's solution was increased more than about 8 mM, neuromuscular transmission was frequently blocked, probably owing to conduction block at nerve branches. In lower concentrations an increase in concentration of potassium ions caused an increase in quantum content (Takeuchi & Takeuchi, unpublished) and the concentration of tubocurarine was increased to block the neuromuscular transmission.

In order to reduce the concentration of chloride, sodium chloride in Ringer's solution was substituted by sodium glutamate. In glutamate Ringer's solution containing 2 mM calcium chloride the muscle fibre had a tendency to contract spontaneously and the amplitude of the e.p.c. showed a large variation. At the time the solution was changed from normal Ringer's solution to glutamate solution contraction occurred frequently, damaging the muscle fibre impaled by the current electrode. This contraction may be due in part to the decrease in resting potential caused by the sudden decrease in chloride ions in outside solution (Hodgkin & Horowicz, 1959*b*), and partly may be due to the decrease in concentration of calcium ion (Fatt & Ginsborg, unpublished observation cited in Boistel & Fatt, 1958). In the present experiments with glutamate Ringer's solution calcium chloride was increased to 5 mM to avoid these disadvantages. Thus glutamate solution contained 10 mM-Cl^-.

Experiments were done at room temperature (18–23° C).

RESULTS

Curarization

When the muscle end-plate membrane, soaked in normal Ringer's solution containing about 3×10^{-6} g/ml. tubocurarine, was clamped at the resting potential by a negative feed-back system, a stimulus applied to the nerve produced an inward current. When a square voltage was applied to the feed-back amplifier, the membrane potential at the point where the electrodes were inserted was suddenly changed to a new level. After a rapid transient current due to charging of the capacitive component of nearby muscle membrane, a residual current flowed through the current electrode, charging the membrane somewhat more remote from the electrode. By changing the amplitude of the applied voltage pulse the membrane potential at the end-plate could be clamped at various potential levels. A stimulus applied to the nerve produced an e.p.c. superimposed on the residual current. Figure 1 shows these currents obtained from a curarized end-plate in normal Ringer's solution. The amplitude of the e.p.c. varied with the value of the clamped membrane potential, the e.p.c.–membrane-potential line crossing the abscissa at about 10–20 mV (inside negative). When the movement artifact was reduced by decreasing the concentration of sodium in the Ringer's solution, the linearity held until after the sign of the e.p.c. was reversed by depolarizing the membrane.

It is known that curarine changes the sensitivity of the end-plate to transmitter. The relation between e.p.c. height and membrane potential obtained from the same end-plate in two different concentrations of tubocurarine is shown in Fig. 2. The slopes of the two lines were different, but the points at which the lines cross the abscissa were almost the same. Since the slope of the line represents the conductance change at the peak of e.p.c., this result shows that tubocurarine decreased the magnitude of the conductance change produced by transmitter, but had little or no influence on other factors which determine the e.p.c. equilibrium potential. From this

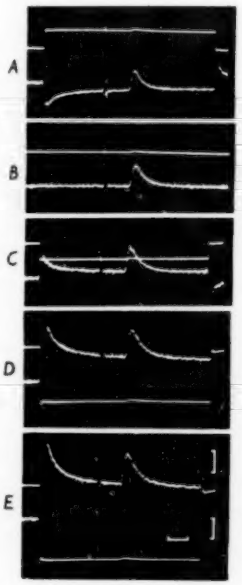

Fig. 1

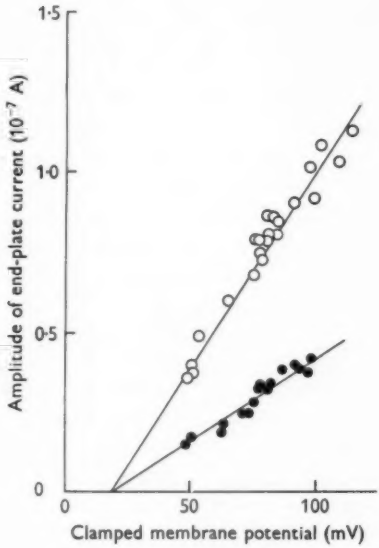

Fig. 2

Fig. 1. End-plate currents and clamped membrane potentials recorded from a curarized end-plate in normal Ringer's solution. Upper traces represent clamped membrane potentials and lower traces feed-back currents containing the end-plate currents. In *A* a square pulse was applied to the feed-back system to depolarize the end-plate membrane. *B*, end-plate current obtained when the membrane was clamped at the resting potential (85 mV). In *C*, *D* and *E* the membrane potential was hyperpolarized to various values. The end-plate currents are superimposed on the current which maintains the membrane potential at various levels. Upper bar, 1×10^{-7}A; lower bar, 10 mV. Time marker, 2 msec. Temperature 20° C.

Fig. 2. Relationship between amplitude of e.p.c. and membrane potential in different concentrations of tubocurarine. Open circles were obtained from an end-plate in 3×10^{-8} g tubocurarine/ml. and filled circles are from same end-plate in 4×10^{-8} g tubocurarine/ml.

result it may be said that when the ionic composition of Ringer's solution was changed, adequate concentration of tubocurarine to block the neuromuscular transmission could be used without influence on the e.p.c. equilibrium potential.

Sodium ions

It has been shown that when sodium concentration in Ringer's solution was decreased, the amplitude of e.p.p. was reduced (Fatt & Katz, 1952), and when the NaCl in Ringer's solution was totally replaced by sucrose, the potential change produced by electrophoretic application of ACh at the end-plate reversed its sign at about -60 mV (del Castillo & Katz, 1955). These findings suggest that sodium ions play an important role in the conductance change produced at the end-plate by the transmitter. In the present experiments the relationship between amplitude of e.p.c. and membrane potential was investigated with various concentrations of sodium ions in Ringer's solution, and differences in the e.p.c. equilibrium potential were estimated by extrapolation. After measurements were done with the end-plate in normal bathing fluid, the potential recording electrode was withdrawn from the muscle fibre to check the resting potential. The current electrode was kept in the muscle fibre to identify the end-plate. Then the solution was changed, and the recording electrode was inserted again at the same end-plate and another set of measurements made. When the recording electrode was inserted immediately after changing to a sodium-deficient sucrose solution, the resting potential was 10–20 mV lower than that in normal Ringer's solution (Giebisch, Kraupp, Pillat & Stormann, 1957). This may be due to the decrease in concentration of external chloride (Hodgkin & Horowicz, 1959*b*). When the preparation was soaked more than 10 min the resting potential returned to the original value, although in some cases the recovery was not complete. Figure 3 presents an example in which the open circles were obtained from curarized end-plate in normal Ringer's solution (Na^+ 113·6 mM), and filled circles were from low-NaCl Ringer's solution (Na^+ 33·6 mM). The e.p.c. equilibrium potentials obtained on extrapolation are separated by about 17 mV. It will be shown that chloride ions had little or no effect on the e.p.c. equilibrium potential. Therefore, since potassium and calcium concentrations in the bathing solution were kept constant, the shift of e.p.c. equilibrium potential should be attributed to the change in concentration of sodium ions. The e.p.c. equilibrium potentials obtained in various sodium concentrations are presented in Fig. 4. Although variations of the e.p.c. equilibrium potential are rather great, there is a definite tendency for the e.p.c. equilibrium potential to increase in sodium-deficient solutions.

When an outward current flows from a NaCl-filled electrode, outflow of Na^+ from the electrode will increase whereas that of Cl^- will decrease

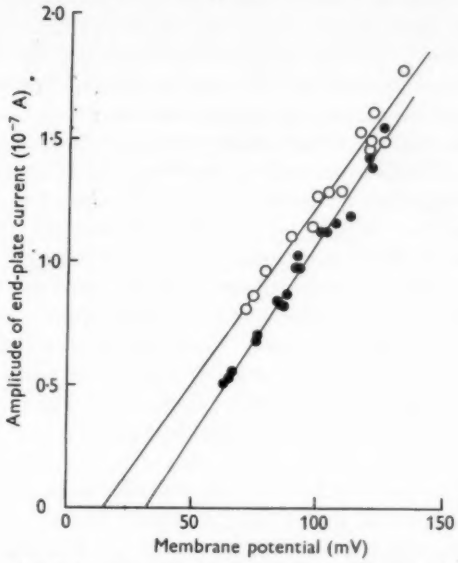

Fig. 3. Relationship between amplitude of e.p.c. and membrane potential obtained from an end-plate in two different concentrations of sodium ion in outside solution. Open circles obtained in normal Ringer's solution (113·6 mM-Na^+) and filled circles in dextrose Ringer's solution containing 33·6 mM-Na^+.

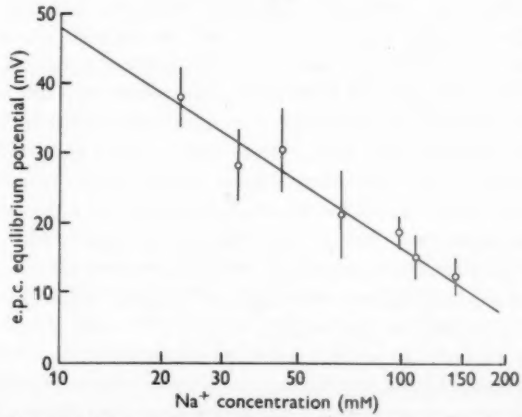

Fig. 4. E.p.c. equilibrium potential plotted against sodium concentration in outside solution on semilogarithmic scale (mean ± standard deviation about the mean). The line is drawn according to $(1/2{\cdot}29)\ \{99 + 1{\cdot}29 \times 58 \log_{10}(15{\cdot}5/Na_o)\}$ mV.

relative to the diffusion of these ions from the electrode in the absence of current. The transfer number of Na^+ through the electrode is not clear, but if it is taken as nearly unity, as suggested by Coombs *et al.* (1955), 1×10^{-8} A of outward current corresponds to 0·1 pequiv/sec of sodium ions. The resting muscle membrane has a relatively low permeability to sodium ions and outward current through the muscle membrane is probably mostly carried by inward-moving chloride ions and outward-moving potassium ions. Thus, sodium ions accumulate within the muscle fibre after a period of outward current passage. The amount of sodium ions accumulating at a point along the muscle fibre should be a function of the total charge.

The change in sodium concentration near the current electrode tip can be calculated as follows. The spreading of sodium ion away from the point where it is injected would be mainly due to diffusion, and the contribution of electrophoretic spread may be neglected. Then the rise in Na concentration after the cessation of the injection at the point where it is injected can be obtained from diffusion equation and by superposition theorem. When 5×10^{-8} A is passed outward through the muscle membrane for 7 min, 210 pequiv of Na ions may be injected. The rise in Na concentration, 100 sec after the cessation of the current, would be 14·5 mM, assuming a fibre diameter of 100 μ and a diffusion constant of 10^{-5} cm^2/sec. This change in internal concentration of Na ions might shift the e.p.c. equilibrium potential by 9·4 mV (see Discussion). At the same time the inside concentration of potassium ions may be decreased and that of chloride ions increased to maintain electrical neutrality.

The effect of the electrophoretic injection of sodium ions into the muscle fibre on the relationship between e.p.c. and membrane potential was investigated with preparations soaked in low-sodium solution. While the outward current from the NaCl electrode was passed into the muscle fibre, the membrane around the electrode was depolarized and the depolarization tended to increase with time. Immediately after the cessation of the current that part of the depolarization associated with the potential drop across the membrane resistance decreased rapidly, and this was followed by slow progressive recovery of the resting potential. The decrease in resting potential after the cessation of outward current may be mostly due to the increase in the inside concentration of chloride ion and partly due to the decrease in potassium ion concentration. The recovery of the resting potential was incomplete within 5 min after termination of the current. The measurements of e.p.c. equilibrium potential were done usually 1–2 min after the cessation of the current. Examples from two different end-plates are presented in Fig. 5. The filled circles represent the e.p.c.–membrane-potential relation before passing current and the open circles after passing current (*A*, 5×10^{-8} A for 7 min; *B*, $4{\cdot}7 \times 10^{-8}$ A for 11 min). After passing

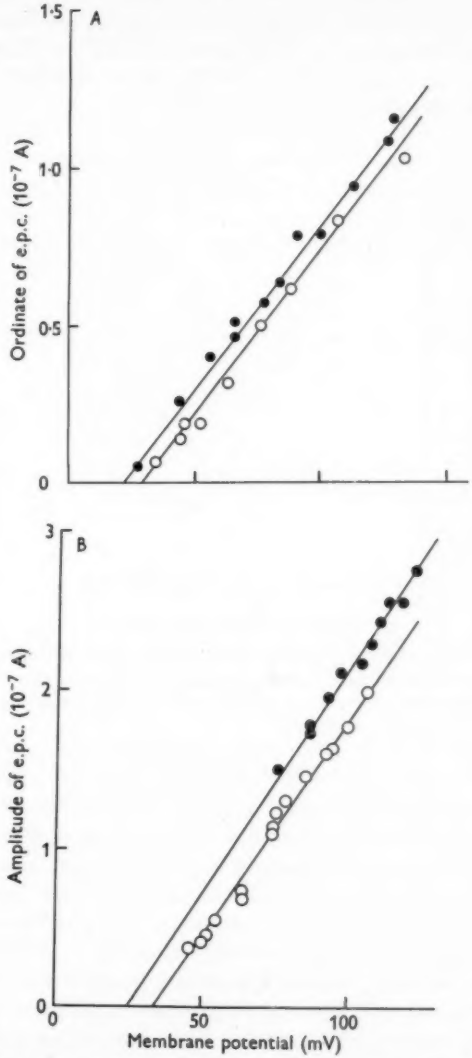

Fig. 5. Effect of sodium injection into muscle fibre at end-plate on the e.p.c.–membrane-potential relation obtained from two different end-plates soaked in Na-deficient Ringer's solution. External Na concentration was 33·6 mM. Filled circles are obtained from end-plates before injection. Open circles in *A* are obtained after electrophoretical injection of sodium ion 5×10^{-8} A for 7 min into muscle fibre, and those in *B* are obtained after injection of $4{\cdot}7 \times 10^{-8}$ A for 11 min.

current the e.p.c.–membrane-potential line was displaced slightly to the right, without appreciable change in the slope of the line. This shift may be attributed to an increase in the inside concentration of sodium, since when outward current was passed from the KCl-filled electrode, the shift in e.p.c. equilibrium potential could not be detected.

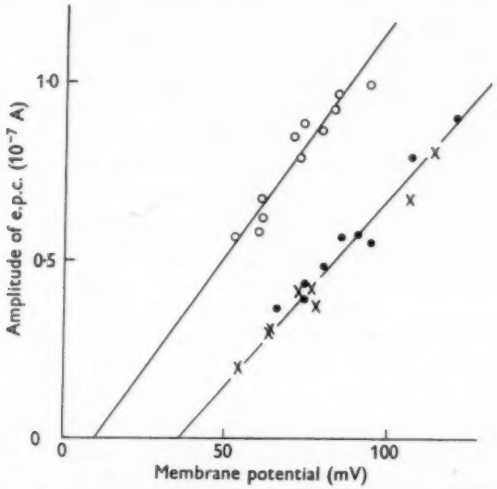

Fig. 6. Effect of potassium concentration on e.p.c.–membrane-potential relation obtained at end-plate. Filled circles obtained in 0·5 mM-K^+ Ringer's solution. Open circles obtained after soaking in 4·5 mM-K^+ solution, and crosses obtained after return to 0·5 mM-K^+ solution.

Potassium ions

The e.p.c.–membrane-potential relation was measured with various potassium concentrations in the range of 0·25–6·8 mM. When the concentration of potassium ions was changed the resting potential and membrane resistance were altered. The change in the membrane resistance, however, had no influence on the e.p.c. under the voltage clamp condition. In Fig. 6 an example is shown in which filled circles represent the relation obtained from an end-plate in low-potassium (0·5 mM) medium, open circles that obtained from the same end-plate in potassium-rich (4·5 mM) medium and crosses that obtained from the same end-plate after returning to low potassium. In this case the e.p.c. equilibrium potential shifted about 28 mV. When the solution was returned to 0·5 mM potassium, the e.p.c. equilibrium potential agreed closely with the initial value. The e.p.c. equilibrium potentials obtained in various potassium concentrations are presented in Fig. 7.

Chloride ions

In order to investigate whether chloride plays some role in the production of e.p.c., chloride was replaced by glutamate, which is probably too large to pass through the membrane, and the e.p.c. equilibrium potential was measured. The resting potential was initially reduced, but recovered gradually to the original value. This decrease in resting potential in low-chloride solutions may be due to the decrease in chloride equilibrium potential and the recovery of the resting potential may be due to restoration of the chloride equilibrium potential as the chloride concentration in

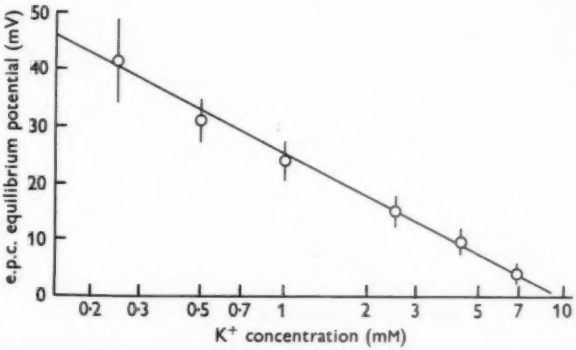

Fig. 7. E.p.c. equilibrium potential plotted against potassium concentration in outside solution on semilogarithmic scale (mean ± S.D.). The line is drawn according to $(1/2{\cdot}29)\{58\log_{10}(126/K_o) - 1{\cdot}29 \times 50\}$ mV.

the muscle fibre decreases. After the muscle is equilibrated with low-chloride solution, and then returned to normal solution, the chloride potential suddenly increases and the resting potential is intermediate between the chloride and potassium potentials. The resting potential then returns to its normal value, as the chloride concentration in the muscle fibre increases (Hodgkin & Horowicz, 1959*b*). In Fig. 8 *A* filled circles represent the relationship in normal solution, while open circles and crosses represent points obtained from the same end-plate 2–3 min and 15 min, respectively, after soaking in glutamate solution. When the outside solution was replaced by glutamate solution the resting potential decreased from 90 to 77 mV, followed by recovery to the original value (86 mV) in about 10 min, indicating a change in the chloride potential; however all lines of the e.p.c.–membrane-potential relation cross the abscissa at about the same point. Similar results were obtained in the muscle equilibrated with low-chloride solution. Open circles in Fig. 8*B* represent the relationship obtained from an end-plate soaked in glutamate solution for about 30 min,

filled circles are those obtained from the same end-plate 2–3 min after returning to normal solution and crosses are those 10 min after returning to normal solution. In this case the resting potential was increased from 69 to 86 mV by bringing the preparation back to normal solution and returned to 77 mV in 10 min, but little or no change in the e.p.c. equilibrium potential was observed. These results suggest that chloride ions contribute

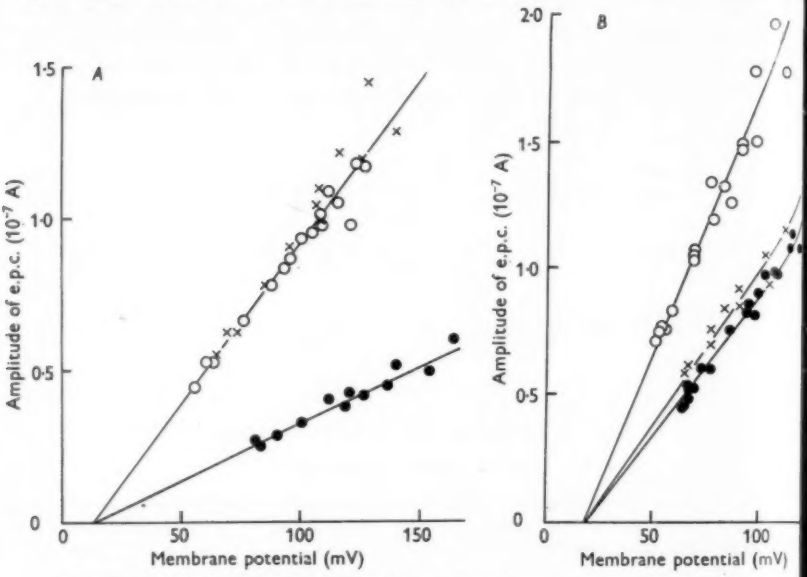

Fig. 8. Effect of chloride concentration on e.p.c.–membrane-potential relation. *A*, filled circles obtained in normal Ringer's solution, open circles show potential after soaking in glutamate Ringer's solution 2–3 min, and crosses after soaking in glutamate solution for 15 min. *B*, open circles obtained from an end-plate equilibrated in glutamate solution, filled circles 2–3 min after bathing in normal solution, crosses 10 min after addition of normal solution. When using glutamate, the concentration of tubocurarine was reduced to $\frac{3}{4}$ or $\frac{2}{3}$ of that in the chloride Ringer, and the calcium concentration increased to 5 mM.

much less to the production of the e.p.c. than do sodium and potassium ions. When chloride ion was replaced by nitrate or sulphate ion, similar results were obtained, i.e. varying the concentrations of these anions had no effect on the e.p.c. equilibrium potential.

DISCUSSION

The potential at which the e.p.c.–membrane-potential line crosses the membrane potential axis is the potential at which the ionic fluxes caused by the transmitter convey no net charge across the membrane. In normal

Ringer's solution this membrane potential was about 15 mV (inside negative). Possible mechanisms of this phenomenon are as follows (del Castillo & Katz, 1956; Katz, 1958): (*a*) the transmitter causes the end-plate membrane to develop a non-selective permeability to free ions on both sides of membrane and there remains the junctional potential between physiological solution and myoplasm (Fatt & Katz, 1951; del Castillo & Katz, 1954), (*b*) the membrane becomes permeable to more than one ion species, e.g. sodium and potassium or sodium and chloride (del Castillo & Katz, 1954) or sodium, potassium, chloride and probably calcium (Nastuk, 1959) and the sum of the ionic fluxes across the membrane becomes zero at −15 mV. In the present experiments the contribution of sodium, potassium and chloride ions to the e.p.c. was tested. The method adopted was to estimate the e.p.c. equilibrium potential by extrapolating the linear relation between e.p.c. amplitude and membrane potential in various solutions. Although this method may have rather large errors because of the extrapolation, a shift of the e.p.c. equilibrium potential could be obtained when the outside concentration of sodium or of potassium was changed. In contrast, no appreciable change could be detected when chloride was replaced by glutamate. When the muscle was soaked in glutamate solution for a long period, the resting potential tended to increase. Although this may suggest that glutamate has some influence on the membrane, e.g. the accumulation of potassium in the cell (Krebs & Eggleston, 1949; Davies & Krebs, 1952), immediately after soaking the preparation in glutamate solution the resting potential decreased, followed by gradual recovery. Thus, immediately after its application, glutamate may be considered as an inert substitute for chloride. In crustacean muscle it is known that glutamate elicits muscle contraction (van Harreveld, 1959) and has a specific action on the muscle membrane at the neuromuscular junction, causing at first depolarization and then desensitization of the membrane to transmitter (van Harreveld & Mendelson, 1959). In frog muscle, however, glutamate had no excitatory action in concentrations as low as that used in crustacean muscle, and the contraction which did occur when most of chloride was substituted by glutamate may be explained by a decrease in chloride equilibrium potential and in calcium concentration. If glutamate neither changes the permeability of the end-plate membrane to other ions nor passes through the end-plate membrane during the action of the transmitter (as is postulated in inhibitory synapses, cf. Shanes (1958)), the present results indicate that as a first approximation the transmitter increases the permeability of the end-plate membrane to sodium and potassium ions but not to chloride ion. There may be other ions to which the end-plate becomes permeable during the action of transmitter, e.g. there is some evidence that, at least in special conditions, permeability of end-plate

membrane to calcium may be increased by action of ACh (Takeuchi & Takeuchi, unpublished). However, in physiological conditions the e.p.c. may be composed of sodium and potassium fluxes and other ions may play only a small role in producing the e.p.c.

The electrical behaviour of the end-plate membrane may be represented approximately by the network shown in Fig. 9. Current can be carried through the membrane by movement of ions through the resistances in the end-plate. The ionic current is divided into components carried by sodium and potassium ions. In a previous report (Takeuchi & Takeuchi, 1959) the electrical behaviour of the end-plate membrane was represented by a series

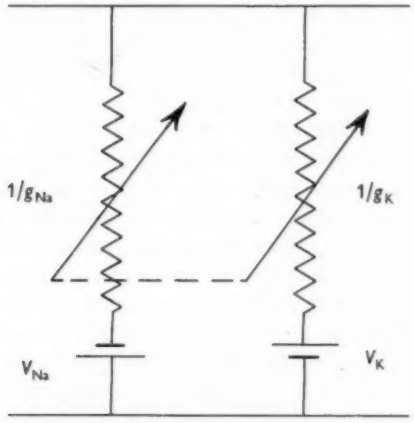

Fig. 9. Electrical diagram of end-plate membrane. For explanation see text.

resistance $R(t)$ and an e.m.f. E where $R(t)$ is a function of time and E is a constant voltage independent of time. Comparing this scheme with that presented in the present report, from the condition that E is independent of time it can be derived that the time course of the changes of resistance in the sodium and potassium channels is the same as that of $R(t)$, i.e. although the resistances in the sodium and potassium channels varied, the ratio of the resistances remains constant.

The results in the present experiments were obtained in the condition that $dV/dt = 0$ during the action of transmitter, where V is membrane potential. Thus the change in the characteristics of the membrane away from the end-plate and the contribution of the membrane capacity may be neglected, since only the change in the end-plate membrane contributes to the change in current. The current carried by potassium and sodium may be simply represented by a conductance and a driving force in each channel.

When the transmitter arrived at the end-plate, the conductances of the sodium and potassium channels increased from their resting values, g°_{Na} and g°_{K}, giving rise to the e.p.c. Thus the e.p.c. may be represented by the following equations:

$$I_{e.p.c.} = \Delta g_{Na}(V - V_{Na}) + \Delta g_{K}(V - V_{K}), \quad (1)$$
$$\Delta g_{Na} = g_{Na} - g^{\circ}_{Na},$$
$$\Delta g_{K} = g_{K} - g^{\circ}_{K},$$

where V_{Na} and V_{K} are equilibrium potential for sodium and potassium, respectively. On rearrangement

$$I_{e.p.c.} = (\Delta g_{Na} + \Delta g_{K}) \left(V - \frac{V_{K} + (\Delta g_{Na}/\Delta g_{K}) V_{Na}}{1 + (\Delta g_{Na}/\Delta g_{K})} \right). \quad (2)$$

$(\Delta g_{Na} + \Delta g_{K})$ corresponds to $1/R(t)$ of previous notation and

$$\left(V_{K} + \frac{\Delta g_{Na}}{\Delta g_{K}} V_{Na} \right) \left(1 + \frac{\Delta g_{Na}}{\Delta g_{K}} \right)^{-1}$$

is e.p.c. equilibrium potential. If the e.p.c. equilibrium potential in normal Ringer's solution is assumed to be -15 mV and V_{K} and V_{Na} are taken as -99 and $+50$ mV, respectively (assuming internal concentration of potassium and sodium ions as 126 and 15·5 mM, respectively, from the data of Boyle & Conway (1941)), $\Delta g_{Na}/\Delta g_{K}$ is 1·29.

If eqn. 2 represents the characteristics of the end-plate response to the transmitter, the equation should predict the changes in e.p.c. equilibrium potential in various ionic conditions. In Figs. 4 and 7 lines are drawn according to the eqn. 2, assuming the ratio $\Delta g_{Na}/\Delta g_{K}$ and the internal concentrations of potassium and sodium are unchanged by altering the external concentrations. Although the experimental data have rather large variations, the observed values agree roughly with the predicted lines. Possible changes in inside concentrations of ions were minimized by soaking the preparation in test solutions for rather short periods (usually 10–20 min). Although the inside concentrations of sodium and potassium were taken from the data of Boyle & Conway (1941) and it is uncertain whether the values are applicable to the present conditions, the coincidence of the values obtained experimentally with those obtained from eqn. 2 support the above assumption. The assumption indicates that at the membrane potential where $V = V_{K}$, e.p.c. may be composed of inward sodium current, at a membrane potential $V_{Na} > V > V_{K}$ e.p.c. may be made of inward sodium and outward potassium fluxes, and at the e.p.c. equilibrium potential the sodium and potassium currents are equal and opposite in direction.

If the transmitter opens new channels to sodium and potassium which are closed in the absence of the transmitter, the e.p.c. equilibrium potential

is the potential at which net current through the end-plate is zero. The equation of Goldman (1943), which has been successfully applied to the resting potential (Hodgkin, 1951; Jenerick, 1953) might be applied to the calculation of the e.p.c. equilibrium potential. But this equation could not describe the shift of e.p.c. equilibrium potential in various ionic conditions, if P_{Na}/P_K was taken as constant. This may suggest that e.p.c. equilibrium potential does not satisfy the conditions for which the equation was derived.

In conclusion it may be said from the present results that the characteristics of the conductance change produced by transmitter at the end-plate membrane are (1) the sodium and potassium conductances increase, with the ratio $\Delta g_{Na}/\Delta g_K$ constant, (2) the amount of the conductance change is independent of the membrane potential, and (3) curarine changes the conductance $(\Delta g_{Na} + \Delta g_K)$, keeping the ratio $\Delta g_{Na}/\Delta g_K$ constant.

SUMMARY

The relationship between the amplitude of end-plate current (e.p.c.) and the membrane potential was investigated under various ionic conditions.

1. In normal Ringer's solution when the e.p.c.–membrane-potential line was extrapolated, the line cut the voltage axis at the point 10–20 mV (inside negative). This point (e.p.c. equilibrium potential) was not changed by altering the curarine concentration.

2. In sodium-deficient solution e.p.c. equilibrium potential became more negative.

3. In potassium-deficient solution the e.p.c. equilibrium potential became more negative.

4. When chloride was replaced by glutamate, no appreciable change in e.p.c. equilibrium potential could be detected, although the resting potential was changed.

5. It may be concluded that, as a first approximation, the transmitter makes the end-plate more permeable to sodium and potassium, but not to chloride.

We wish to express our sincere thanks to Drs C. C. Hunt, A. R. Martin, and C. Edwards for their constant advice and criticism.

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MONOSYNAPTIC EXCITATORY ACTION ON MOTONEURONES REGENERATED TO ANTAGONISTIC MUSCLES

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There have been many attempts to discover if the transformation of reflex function by tendon transposition or by nerve cross-union effects any change in the simplest reflex pathways. A comprehensive and critical review of the literature led Sperry (1945) to conclude that there was little, if any, acceptable evidence that such a 'plastic' change occurred in mammals, even when very young, a conclusion supported by more recent investigations (Sperry, 1947; McIntyre & Robinson, 1958).

In contrast, there are many accounts of remarkable plastic changes in reflex phenomena following various operative procedures, including nerve cross-union, on teleosts and larval amphibians. These experimental investigations have generally been concerned with complex reactions such as movements of whole limbs (Sperry, 1950; Sperry & Deupree, 1956) or of the jaw (Arora & Sperry, 1957), and not with the contractions of isolated muscles. Under such conditions unrecognized feed-back mechanisms are likely to distort the responses and greatly to complicate their interpretation. Moreover, such movements merely signal the net result of the contractions of the antagonistic muscles controlling movement at each joint; hence it is hazardous to use such observations in establishing that there have been changes in the synaptic connexions on to individual nerve cells. Nevertheless, these investigations form a striking contrast to comparable mammalian experiments and suggest that the central nervous systems of teleosts and larval amphibians have well developed plastic properties. The experiments on mammals have given no indication of such plastic changes in the central nervous system (Sperry, 1941, 1942, 1945, 1947; McIntyre & Robinson, 1958); and it has been suggested by Sperry that embryonic mammals may have a comparable plasticity, which is already lost about the time of birth.

Hitherto there has been no investigation of the possibility of plastic modification of monosynaptic pathways from primary afferent fibres to motoneurones, which are now particularly well suited to quantitative

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evaluation. A fairly precise measure of the monosynaptic activation of a motoneurone is provided by the size of the excitatory post-synaptic potential (EPSP) as recorded by an intracellular micro-electrode. In this way it has been possible to define the pattern of monosynaptic activation for over a thousand motoneurones supplying the hind limb and forelimb muscles of the cat (Eccles, Eccles & Lundberg, 1957*b*; R. M. Eccles & Lundberg, 1958). With rare exceptions, it has been found that the monosynaptic activation from the afferent fibres (Group Ia) of any particular muscle operating at the knee or ankle joints is restricted to the motoneurones concerned in the movements subserved by that muscle, i.e. to the motoneurones of that muscle and of synergic muscles. A systematic investigation of the monosynaptic EPSPs thus provides the most delicate possible test for any change in central pathways that might result from an inversion of motoneurone function consequent on nerve cross-union. For example, if the function of a motoneurone normally innervating a pre-tibial muscle is changed by the regeneration of its axon to an ankle extensor muscle, intracellular recording from this motoneurone would be able to detect very small developments of monosynaptic innervation from the afferent fibres of other ankle extensor muscles.

METHODS

Factors in the design of the experiment

Several factors have to be taken into account in choosing muscle nerves for the cross-union operation (cf. Fig. 1A): (1) the two nerves N_1 and N_2 must be so placed that the cross-union operation is anatomically possible and they must supply muscles (M_1 and M_2) that are not synergists and preferably are antagonists. (2) M_1 and M_2 must each have one or more synergic muscles MS_1 and MS_2. The nerves NS_1 and NS_2 of MS_1 and MS_2 are not molested in the initial operative cross-union, and hence their afferent fibres provide the control afferent pathways that are employed in testing the motoneurones with changed functions. (3) As a rule afferent volleys in NS_1 or NS_2 will normally evoke considerable monosynaptic EPSPs in the respective synergic group of motoneurones, MN_1 or MN_2, which is being subjected to the cross-union procedure, i.e. these volleys will produce heteronymous EPSPs (cf. Eccles *et al.* 1957*b*). It is very important, however, that monosynaptic EPSPs should very rarely be produced normally by NS_1 volleys on the motoneurones of group MN_2 or by NS_2 volleys in MN_1, because, in the interpretation of the experimental results, such aberrant EPSPs (Eccles *et al.* 1957*b*, p. 41) provide the criterion for the establishment of new connexions as a consequence of the cross-union between N_1 and N_2. The sensitivity of this criterion would be reduced if such aberrant responses were commonly observed in the control experiments in which there was no operative cross-union. (4) Finally our experiments have indicated that it is very important for the motoneurones with changed function (say MN_1) to be in close proximity to the motoneurones (MNS_2) belonging to the antagonist test nerve (NS_2 in this case).

It is possible to design several experiments on the cat's hind limb that conform reasonably closely with these specifications. In eleven kittens, from 1 to 25 days old, the nerve to the peroneal muscles, peroneus longus, brevis and tertius (Per), was cross-united to the nerve to the medial gastrocnemius (MG), as illustrated in Figs. 1B, 2B. The deep peroneal (DP) nerve to the remaining pre-tibial muscles, and the nerves to the remaining post-tibial

extensor muscles—lateral gastrocnemius-soleus (LG), plantaris (Pl) and flexor digitorum longus (FDL)—serve as the respective control afferent pathways. Cross-unions were also performed in six kittens between the Per and LG nerves. More recently another cross-union has been attempted, DP to MG, but some months must elapse before the final experimental tests.

None of these experiments provides ideal conditions. For example, in the LG-to-Per cross-union the motoneuronal nuclei belonging to the two testing nerves, MG and DP, are

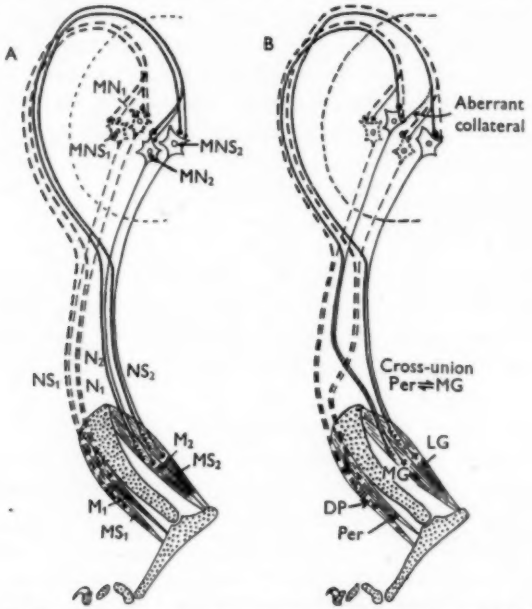

Fig. 1A. Diagram showing the monosynaptic pathways for two pretibial muscles and two post-tibial muscles, labelled in accordance with the text description. Only one Group Ia afferent fibre and one motor fibre is drawn for each muscle, the afferent fibres being distinguished by their thicker lines. The afferent and efferent pathways for muscles functioning as flexors are shown by broken lines. It will be seen that the afferent fibres have monosynaptic action on the motoneurone of their own muscle and, to a less extent, on that of the synergic muscle, as shown by the thinner collateral branch. B. Diagram showing changed peripheral connexions when regeneration has followed the cross-union. The change in function is shown by the change in broken line designation. In the spinal cord the dotted line shows the aberrant connexions that developed from the Ia afferent fibres of post-tibial extensors to the motoneurones of the peroneal muscles when nerves to medial gastrocnemius and to the peroneal muscles were cross-united.

each some distance from the respective nuclei under test, the MG nucleus being caudal to the Per motor nucleus; while the DP nucleus is a considerable distance dorsolateral to the LG nucleus and somewhat more rostral (cf. Romanes, 1951). The Per-to-MG cross-union provides one test situation of close proximity; the Per and LG nuclei are juxtaposed at the same segmental level (L7), the Per nucleus being just lateral to that for LG. The other

test situation, MG-to-DP, is most unfavourable, as the respective nuclei are at different segmental levels, and there is also considerable transverse separation. As a consequence our experimental testing has been concentrated on Per motoneurones that have regenerated to innervate the MG muscle, as in Fig. 1B. This situation has the disadvantage that the Per muscles make a negligible contribution to ankle flexion (peroneus brevis being in some ankle positions even a very weak extensor), which may presumably be correlated with the normal occurrence of a rather high proportion of aberrant monosynaptic excitatory connexions from some post-tibial extensors to Per motoneurones (cf. particularly from MG; Table 2). However, most Per motoneurones have synaptic connexions that indicate not only synergism with the DP motoneurones (monosynaptic EPSPs from DP nerve), but also antagonism to the post-tibial extensors (Ia IPSPs from one or other of these). It was surprising to find that the frequency of Ia IPSPs from post-tibial extensor muscles was as high (22/49) for Per motoneurones as it was for the DP motoneurones supplying the much more effective antagonists (25/56). An earlier investigation (Eccles *et al.* 1957*b*, Table 4) showed that peroneus longus motoneurones received much more monosynaptic excitation from the DP nerve than did the other peroneus motoneurones. In the present investigation it was not possible to discriminate between the three peroneal muscles, because the nerve to all three was cross-united to the MG muscle.

Experimental procedures

The nerve cross-union was performed in an initial aseptic operation. In order to prevent the nerves growing back into their own muscles, the two nerves to be crossed were severed as far distally as possible, and the central ends were freed as far proximally as was necessary to allow suturing in the crossed position without undue tension (cf. Fig. 2B). A single strand of nylon thread was employed to tie the cut ends together and the sheaths of both stumps were torn open by fine forceps so that nerve regeneration would not be impeded. In the final experimental tests 21 to 38 weeks after the cross-union, it was invariably found that the muscles were appropriately innervated and that there were no aberrant connexions to other mucles. For example, in the main series of Per-to-MG cross-union, the Per nerve innervated only MG and the MG nerve only the Per muscles. At the end of the intracellular investigation the various nerves, MG, LG and peroneal, were dissected from the sciatic and stimulated. The responses were recorded from the peripheral nerves that had been employed for stimulation during the experiment. Again it was found that there was no evidence of even one stray nerve fibre. For example, with the cross-union described above, the nerve innervating MG muscle had all its fibres in the peroneal nerve, while the nerve innervating the Per muscles had all its fibres in the MG nerve.

The experimental technique in the final experiment resembled that already described for a survey of the monosynaptic excitatory fields of normal motoneurones (Eccles *et al.* 1957*b*). The cross-united nerves were severed approximately at the site of the suture and the central ends were dissected free sufficiently far to enable the cathode of the stimulating electrodes to be applied well proximal thereto. Since the micro-electrodes were usually filled with 3 M-KCl, IPSPs were sometimes inverted to depolarizing potentials on account of the diffusion of Cl^- out of the electrode. Such inverted IPSPs were readily distinguished from EPSPs by their conversion back to hyperpolarizing potentials when a steady depolarizing current was passed through the micro-electrode. At the end of each intracellular investigation of a motoneurone the micro-electrode was withdrawn to a position just extracellular to the particular neurone and the potentials produced by the various nerve volleys were again recorded. The potential changes actually occurring across the cell membrane are given by the difference between the intracellular and the corresponding extracellular record. As is shown in the illustrative figures, each record is made by the superposition of many faint traces, much of the background noise being thus rejected. Post-tetanic potentiation was also employed in order to lift the post-synaptic potentials above the noise level. The conditioning tetanus was standardized at 450/sec for 15 sec.

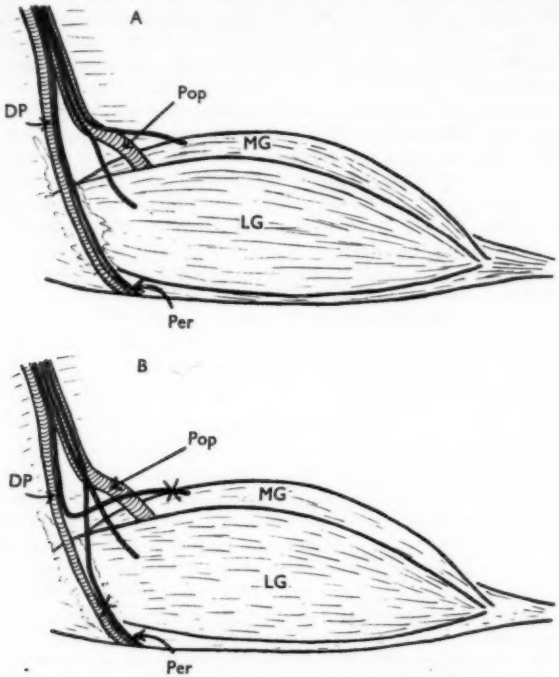

Fig. 2. Drawing of the field of operation when the nerve to the peroneal muscles is cross-united to the medial gastrocnemius nerve, A being before and B after the cross-union, the crosses showing the positions of the sutures. MG, LG mark the positions of the medial gastrocnemius and lateral gastrocnemius muscles and Per labels the nerve to the peroneal muscles; DP is the deep peroneal nerve supplying tibialis anterior, extensor digitorum longus and extensor digitorum brevis muscles, and Pop is the popliteal nerve.

RESULTS

The ventral roots were left intact so that each motoneurone penetrated by the micro-electrode could be identified by antidromic invasion from its motor axon (cf. Eccles *et al.* 1957*b*). For example, in Fig. 3A the stimulus to the Per nerve (re-innervating the MG muscle) was just at threshold for the motor axon of the cell. In about half the superimposed traces this was excited, giving the large truncated spike-potential, which is seen in full at the much lower amplification of the traces in 3E. In the remaining half of the traces in Fig. 3A the monosynaptic EPSP alone was generated. The time course of the potential change across the cell membrane was obtained by projecting and tracing so as to superimpose on the intracellular record the extracellular record (the third line of A) taken at the same stimulus

strength and immediately after withdrawal of the electrode from the cell. The difference between the two traces gives the time course of the membrane potential change, and is shown below each set of recordings of Fig. 3A–D and F–I.

In this case of reinnervation of MG muscle by the Per nerve it is immediately evident that depolarizing potentials with the brief latency characteristic of monosynaptic EPSPs were set up by LG, Pl and FDL nerves in

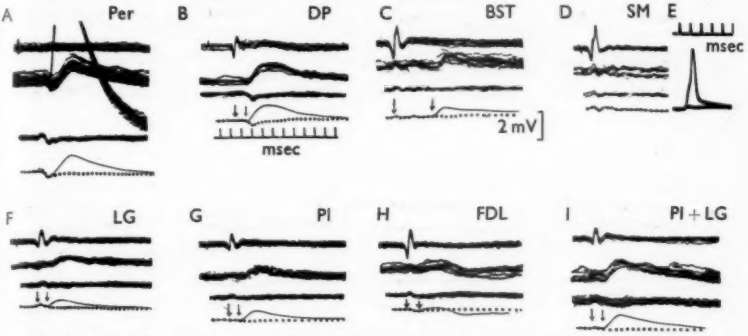

Fig. 3. Intracellular responses of a Per motoneurone reinnervating MG muscle. The three records of each ensemble A–D and F–I are formed by the superposition of about twenty faint traces and show, from above downwards: the potential produced by an afferent volley at the dorsal root entry at mid L 7 segmental level, negativity being upwards; the intracellular potential of a Per motoneurone having a membrane potential of −75 mV and spike potential of 109 mV, positivity being upwards; the potential produced by the same volley, but with a just extracellular position of the micro-electrode. Below the three records there is a superimposed tracing of the intracellular (continuous line) and extracellular (dotted line) records showing the time course of the potential change across the neuronal membrane. With A the stimulus to the Per nerve was just at threshold for the axon of the motoneurone, producing an antidromic spike potential in about half the intracellular traces (middle series), which is shown at much lower amplification in E. B–D and F–I were produced by a maximum Group I afferent volley in nerves indicated by the symbols; DP, deep peroneal; BST, posterior biceps-semitendinosus; SM, semimembranosus + anterior biceps; LG, lateral gastrocnemius + soleus; Pl, plantaris; FDL, flexor digitorum longus. With Pl + LG the two volleys were synchronized. Same time scale for all records and same potential scale for all intracellular and extracellular records except E. The two arrows on the superimposed traces give the central synaptic delay.

F, G and H respectively, in addition to the EPSPs set up by the Per and DP volleys in A and B respectively. On the other hand, in C and D, BST and SM volleys failed to set up monosynaptic EPSPs. The EPSP produced by the LG plus Pl volleys (I) was nearly as large as that generated homonymously in A or by the afferents from the normal synergic muscles in B.

Enhancement of the EPSPs by post-tetanic potentiation was regularly employed in order to increase the size of the EPSPs, and was particularly

valuable in attempting to lift very small EPSPs above the noise level so that they could be identified and their latency and subsequent time course determined. In Fig. 4 the records in the rows marked Con and PTP are respectively the control and the most potentiated. In every case stimuli maximal for Group I were applied to the afferent nerve. Besides the Ia EPSPs, polysynaptic EPSPs are shown in C, G and D, H to be potentiated,

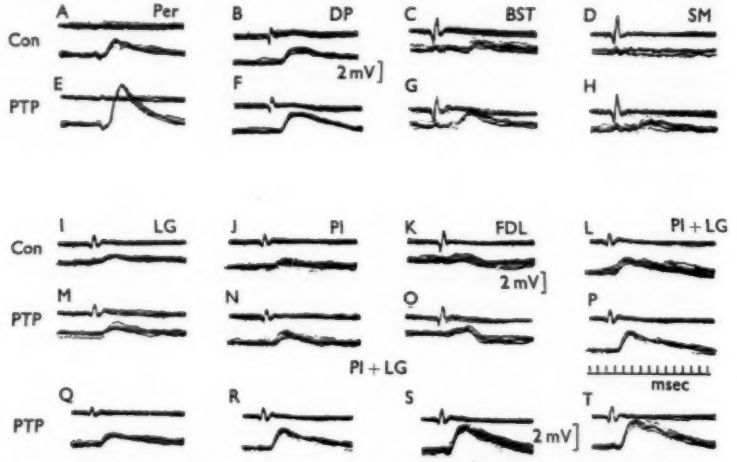

Fig. 4. Each pair of records of A–P corresponds to the upper pair of each ensemble in Fig. 3, and is from the same Per motoneurone, with the same afferent nerve identification. In addition they are arranged in rows labelled Con and PTP, which are respectively the control EPSPs and those at the height of the post-tetanic potentiation following stimulation at 450/sec for 15 sec. Q–T show responses to PI + LG volleys at the height of post-tetanic potentiation, being evoked by stimuli of increasing strength from Q to T, Q being so weak that it was submaximal for Group Ia and below the Ib threshold. Same time scale for all intracellular records; Q–T being at higher amplification than A–P, as shown by the respective potential scales.

while in K, O the polysynaptic IPSP was also potentiated. This Per motoneurone thus received a considerable monosynaptic excitation from the nerves to the post-tibial extensors (I–P), an aberrant effect of a size never observed in normal Per motoneurones. The series with graded stimulus strengths Q–T shows that this monosynaptic EPSP was produced by the lowest threshold Group I afferent fibres (cf. also Fig. 5A–C, G–K), and hence is attributable to the Group Ia fibres (cf. Eccles, Eccles & Lundberg, 1957*a*, *b*).

The hyperpolarizing IPSPs in Fig. 4K and O reveal that the IPSPs of this motoneurone had not been reversed by the diffusion of Cl^- ions out of the KCl electrode (cf. Coombs, Eccles & Fatt, 1955). All membrane

depolarizations in Figs. 3 and 4 would thus be EPSPs. However, in many experiments Cl^- diffusion had converted IPSPs to depolarizing potentials, and it was necessary to depolarize the motoneurone by the application of

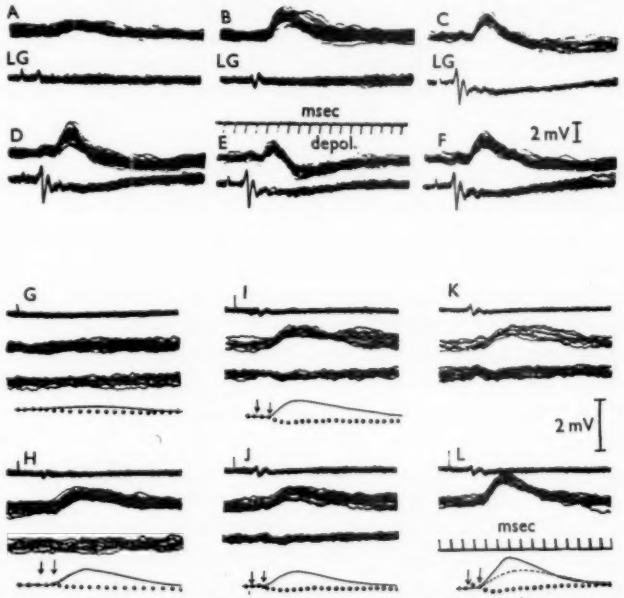

Fig. 5. A–G. Upper tracings are intracellular records from another Per motoneurone reinnervating MG muscle (membrane potential −55 mV), responses being evoked by LG volleys, the lower tracings being of the incoming afferent volley at the mid L 7 level. The LG stimulus was just above the Ia threshold in A, and submaximal and maximal for Group I in B and C respectively. D–F were evoked by a maximum Group I volley, the neuronal membrane being depolarized in E by passage of a steady current through the micro-electrode. Note in E the large hyperpolarization of the IPSP which cuts into the rising phase of the monosynaptic EPSP, an effect which is much less obvious in the control records D and F.

G–L are intracellular records from another Per motoneurone reinnervating MG muscle (membrane potential −60 mV, spike potential 79 mV), the responses evoked by LG volleys, which in G–K are set up by progressively increasing strengths of stimulation as in A–C, J being maximal for Group Ia and K for group I. L shows effect of post-tetanic potentiation of EPSP set up by a maximal Group I volley. Superimposed traces of intracellular and extracellular records reveal the time course of the membrane potential; the two arrows give approximately the central synaptic delay.

a current through the micro-electrode in order to convert IPSPs to hyperpolarizing potentials (Fig. 5 D–E) and so to discriminate them from EPSPs (cf. Eccles, Eccles & Lundberg, 1957*c*). The IPSPs invariably had a central latency in excess of 1·3 msec (Eccles *et al.* 1957*c*), whereas the

latency of normally occurring EPSPs was not longer than 1·0 msec (cf. Figs. 3B, 4B, 7F).

EPSPs generated in Per motoneurones by volleys in the nerves of the post-tibial extensor muscles have often had central latencies in the range 1·0–1·3 msec. The small size of most of these EPSPs would result in a rather late estimate being made of their origin in time, and hence account, at least in part, for such high figures for monosynaptic latency. But even apart from this factor the latencies appear to be abnormally long. All transitions exist between undoubted monosynaptic EPSPs and EPSPs with latencies in excess of 1·5 msec (Fig. 7H, I), which are certainly due to Ib disynaptic action. In practice all EPSPs with a latency in excess of 1·3 msec have been regarded as not being monosynaptically evoked. The second criterion has been the threshold discrimination between Ia and Ib afferent fibres. Even when there has been no appreciable difference of Ia and Ib conduction velocity, afferent volleys evoked by stimuli up to 1·2 times threshold only produce monosynaptic Ia EPSPs in motoneurones (Eccles *et al.* 1957*c*). As is shown in Figs. 4Q–S, 5A–C, G–K, the lowest threshold afferent fibres in the nerves to the post-tibial extensor muscles were responsible for the brief-latency EPSPs in Per motoneurones reinnervating MG muscle. It has always been found that the lowest threshold (Ia) afferent fibres were responsible for the monosynaptic EPSPs in the Per motoneurones with changed function, which is precisely the situation for the EPSPs of normal motoneurones (Eccles *et al.* 1957*a*).

Altogether, in the eleven experiments, satisfactory intracellular investigations were made on 102 Per motoneurones which had reinnervated MG muscle. In addition 59 normal Per motoneurones in normal cats were investigated in order to give a contro series. The entries for Per motoneurones in the second rows of Tables 1 and 2 are derived from these two series. The monosynaptic EPSPs of the other species of motoneurones involved in the cross-union (MG) or synergic with those cross-united (DP and LG) are given also in the other rows of the tables. The entries in each column relate to the monosynaptic EPSPs produced by maximum Group I volleys in the various muscle nerves indicated by the letters at the top. Each series of entries for the action of each species of nerve on each species of motoneurone gives, first, the number in which monosynaptic EPSPs were observed out of the total number of motoneurones so investigated, and secondly the mean EPSP calculated by dividing the aggregate EPSPs by the total number of those motoneurones. For example, the aggregate EPSP for the action of LG volleys on Per motoneurones was 20·3 mV, which gives a mean of 0·20 mV for the 102 motoneurones so investigated.

Comparison of the Per motoneurones in Tables 1 and 2 reveals that the

control nerves to the post-tibial extensor muscles evoked monosynaptic EPSPs far more frequently in the Per motoneurones with changed function (see and compare framed sections of each Table), the increase being

TABLE 1. Assembled results for the eleven experiments with cross-union of Per with MG. Maximum monosynaptic EPSPs in motoneurones (arranged in rows) by volleys in eight different muscle nerves (arranged in columns). The upper entry gives the number of cells responding by recognizable monosynaptic EPSPs over the total so investigated. The lower entry gives the mean EPSP for the total cells so investigated. Cells with very low membrane potentials (less than −40 mV) were not included in this Table

	Nerve volleys							
Motoneurones	DP	Per	MG	LG	PL	FDL	BST	SM
DP	107	48	0	2	3	8	0	0
	107	107	107	107	107	102	107	94
(mV)	2·96	0·22	0	0·01	0·01	0·03	0	0
Per	92	85	3	41	17	23	2	0
	102	100	101	102	101	95	101	91
(mV)	1·50	1·17	0·03	0·20	0·05	0·08	0·01	0
MG	1	2	31	31	2	0	0	0
	31	31	32	33	31	28	31	26
(mV)	0·02	0·07	2·1	2·05	0·01	0	0	0
LG	0	1	25	31	18	0	1	1
	31	31	31	31	31	29	31	22
(mV)	0	0·01	0·59	3·6	0·84	0	0·03	0·01

In both tables the abbreviations for nerves and motoneurones are as follows: DP, deep peroneal; Per, peroneus longus, brevis and tertius; MG, medial gastrocnemius; LG, lateral gastrocnemius + soleus; Pl, plantaris; FDL, flexor digitorum longus + flexor hallucis longus; BST, posterior biceps plus semitendinosus; SM, semimembranosus + anterior biceps.

TABLE 2. Assembled results from twelve normal cats, tabulated identically with Table 1. Note the contrast between the corresponding entries which are framed in the two Tables

	Nerve volleys							
Motoneurones	DP	PER	MG	LG	PL	FDL	BST	SM
DP	69	35	0	0	0	1	0	0
	69	70	51	51	52	51	42	17
(mV)	3·90	0·90	0	0	0	0·01	0	0
PER	40	59	8	3	1	6	0	0
	56	59	53	52	54	54	42	29
(mV)	0·94	3·66	0·08	0·015	0·005	0·04	0	0
MG	0	1	42	43	16	5	0	0
	27	24	42	43	40	33	27	16
(mV)	0	0·01	4·85	2·04	0·20	0·06	0	0
LG	0	1	34	34	33	4	0	0
	25	20	34	34	34	32	24	14
(mV)	0	0·05	2·27	4·7	1·1	0·02	0	0

particularly large with the LG nerve (from 6 to 40 %), but also considerable with Pl and FDL motoneurones, as is seen in Figs. 3 and 4. This is associated with an increase in the mean size of the monosynaptic EPSPs produced

by LG volleys in Per motoneurones by more then tenfold, from the normal value of about 0·015 mV to 0·20 mV for the Per motoneurones with changed function, and there were also large increases in EPSPs evoked by the Pl and FDL afferent volleys. Usually the LG volleys were the most effective, as is illustrated in Figs. 6 and 7. Comparison of Tables 1 and 2 shows further that there was virtually no alteration in the monosynaptic action of volleys in DP, BST and SM nerves on the Per motoneurones with changed function (cf. Figs. 3, 6, 7; Tables 1 and 2).

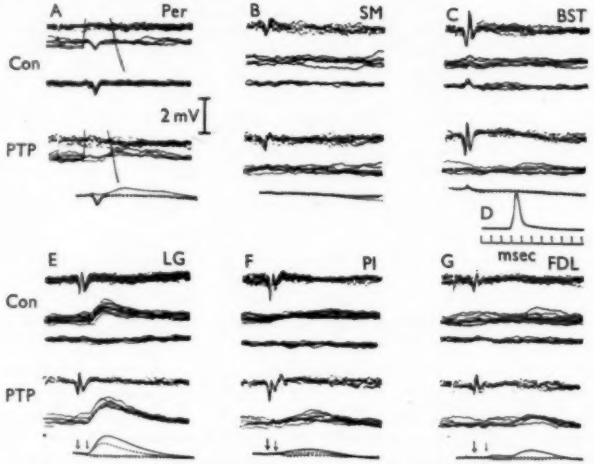

Fig. 6. A–C and E–G show both intracellular and extracellular records from a Per motoneurone that reinnervated MG muscle (membrane potential −76 mV, spike potential 76 mV), arranged to show intracellular records before (row labelled Con) and after post-tetanic potentiation (row labelled PTP). Bottom trace of Con in each ensemble is the corresponding extracellular record; below PTP are the superimposed tracings of the two intracellular (broken line, Con; continuous, PTP) and the extracellular (dotted line) records, the arrows giving the approximate central synaptic delays. The afferent volleys are identified as in Fig. 3. D shows the intracellular antidromic spike evoked by a Per volley, and recorded at much lower amplification. Same time scale for all records, and all but D at same amplification. The afferent volley records are at mid L7 level, negativity being upwards.

Finally, both the MG and Per nerves had diminished actions on the Per motoneurones in Table 1 as compared with the control actions in Table 2. This diminution is also evident for the actions of these two nerves on the other motoneurones of Tables 1 and 2. Undoubtedly the principal factor concerned in this effect is the chromatolytic degeneration of a considerable proportion of the Group I afferent fibres of Per and MG nerves. Chromatolysis, both of spinal ganglion cells and of motoneurones, is particularly severe after peripheral nerve section in young animals, and

it has been reported that it causes the death of a very large proportion of these nerve cells (Nonidez, 1944; Romanes, 1946). Correspondingly, the surface lead from the dorsal root at cord entry shows only a very small spike for afferent volleys in Per and MG nerves (Figs. 3A; 4A; 6A; 7A, B). In a few experiments monophasic recording from dorsal roots was employed in estimating the proportion of surviving Group I afferent fibres in the cross-united nerves, the values being in the range of 20–40 %. The depressions of monosynaptic excitatory actions in Table 1 as compared with

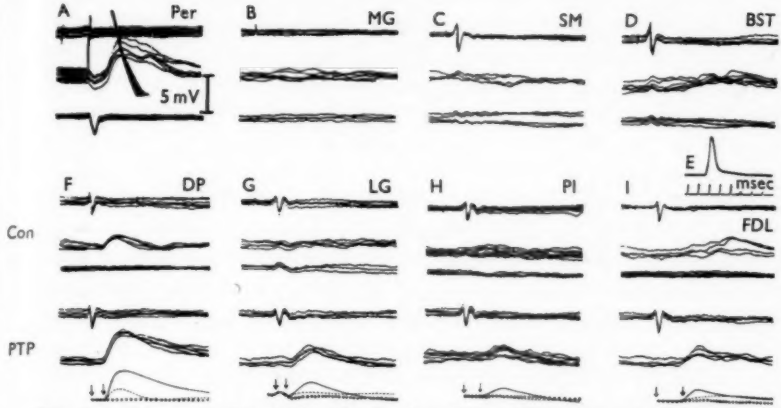

Fig. 7. Series as in Fig. 6, but for another Per motoneurone reinnervating MG muscle (membrane potential −74 mV, spike potential 82 mV). Post-tetanic potentiation is only shown for F–I. The afferent volley records are from the mid L7 level, negativity being downwards.

Table 2 accord well with this proportion of survival. Thus the homonymous action of the Per afferent volley is depressed to 0·31 (1·17:3·66) and the heteronymous action on DP motoneurones to 0·24 (0·22:0·90), while for the MG volley the corresponding homonymous and heteronymous ratios are 0·43 (2·1:4·85) and 0·26 (0·59:2·27). Presumably the reduction of the aberrant MG action on Per motoneurones to about one third (0·03:0·08) may be accounted for in the same way.

The post-tetanic potentiation of EPSPs following a long high-frequency tetanus (Eccles, Krnjević & Miledi, 1959; Curtis & Eccles, 1960) has been employed regularly in defining more accurately the time course of small EPSPs, especially in the measurement of central latency. It has been of particular importance when there was uncertainty about the presence of an EPSP. Post-tetanic potentiation would then either increase the EPSP so that its existence was undoubted (Fig. 6, F, G; Fig. 7, G), or alternatively would show that the EPSP had such a long latency that it was not monosynaptic; e.g. it was shown in this way to be 1·5 and 2·5 msec

in Fig. 7H and I respectively. Sometimes the relative potentiation has been abnormally high (3·8 in Fig. 7G) but often the EPSPs produced by the post-tibial extensor nerves in Per motoneurones were not potentiated more than were EPSPs from the DP nerve to Per motoneurones, as may be seen by comparing in Fig. 4, B with F, I with M, J with N and L with P. With the Per motoneurones of Table 1 the mean potentiation ratio was 2·5–2·6 for monosynaptic EPSPs produced by nerves of the post-tibial extensors, and about 2·15 for the DP nerve. Rarely, post-tetanic potentiation revealed a monosynaptic EPSP that was otherwise undetectable, there being for example three instances of this in the 41 EPSPs reported for LG afferent volleys on Per motoneurones in Table 1. As a result of the relatively high degree of post-tetanic potentiation, the mean monosynaptic EPSP for the combined action of the nerves to the post-tibial extensors was almost 1·0 mV for the 102 Per motoneurones which had been changed to function as ankle extensor motoneurones.

The responses of MG cells in Table 1 show little, if any, increase in aberrant connexions from the acquired synergists, the muscles innervated by DP nerve, and are thus in striking contrast with the responses of Per cells. A similar absence of aberrancy from nerves to the acquired synergists was observed when LG and Per nerves were cross-united in six kittens. In a total of 21 LG and 5 Per motoneurones there were no aberrant monosynaptic connexions. It was noteworthy that not only had the cells retained their original monosynaptic activation by Group Ia afferent fibres, but also that the Ia disynaptic inhibitory actions on them from the original antagonists (the acquired synergists) were at least as frequent as in the control investigations.

The negative results of such cross-union operations account for the very low proportion of aberrancy (4/50) reported in a preliminary account of this present investigation (Eccles, 1959). Changed function in these 50 motoneurones resulted from the LG–Per cross-union in 26, and from the MG–Per cross in 24. Of these latter only 10 were Per motoneurones; they displayed much the same proportion of aberrancy as the much larger series of Table 1, 4/10 for LG and 1/10 for Pl.

Hitherto attention has been restricted to the evidence that change in motoneurone function by operative cross-union results in a change of the receptive field from which the motoneurone draws its monosynaptic activation. But operative cross-union also entails a change of the muscle from which an afferent fibre receives its activation. For example, as a consequence of the nerve cross-union of Table 1, the Per afferent fibres were excited by receptors in MG muscle and the MG afferent fibres by receptors in the Per muscles (cf. McIntyre & Robinson, 1958). Comparison of Tables 1 and 2 shows that, as a result of this changed peripheral con-

nexion, there was no development of central connexions, of these afferent fibres to the motoneurones of their acquired synergists, from the Per and MG nerves to LG and DP motoneurones respectively. There was merely a diminution in the sizes of the EPSPs normally produced by both afferents, which, as we have seen, is sufficiently explained by the chromatolytic death rate.

DISCUSSION

In general, the experimental results as presented in Tables 1 and 2 show that after birth the mammalian spinal cord has only a small degree of plasticity, in so far as this is exhibited by changes of monosynaptic reflex pathways following cross-union of nerves to muscles. For example, in two experiments there was no trace of any aberrant connexions to the 23 Per motoneurones that had reinnervated MG muscle. In three other experiments there were aberrant connexions to more than half of the Per motoneurones with changed function. Yet the mean values for the aberrant EPSPs produced in Per motoneurones from the various nerves to the post-tibial extensors (LG, 0·5 mV; Pl, 0·3 mV; FDL, 0·4 mV) show that the formation of new synaptic connexions usually occurred only to a slight extent. There was no correlation between the age of the kitten at operation (range 1–25 days) and the frequency and magnitude of the aberrant EPSPs.

The present experimental evidence of a small degree of plastic change in the synaptic connexions of the mammalian spinal cord is not in conflict with the negative results of earlier investigations. For example, the small changes here reported in the Ia receptive fields of motoneurones would not be detectable in investigations on reflex discharges in ventral roots (McIntyre & Robinson, 1958) or in reflex responses of limbs (Sperry, 1941, 1942, 1947).

The aberrant EPSPs have tended to differ from normal EPSPs in two respects, longer central latency and larger post-tetanic potentiation. The longer latency might be expected to occur on account of increased conduction time in the newly developed presynaptic fibres. Presumably also the newly developed synaptic terminals may be relatively ineffective liberators of the chemical transmitter, and hence show more potentiation as a consequence of the transmitter mobilization ensuing after a conditioning tetanus (cf. Curtis & Eccles, 1960). The mean PTP ratio for the aberrant EPSPs, 2·6, was much higher than the normal ratio of 1·5:2·0. It may be of significance that there was also an abnormally high level of potentiation for the EPSPs evoked by DP and P volleys on Per motoneurones, which had mean ratios of 2·1:2·5 respectively; for it will later be suggested that in part such EPSPs were also produced by newly developed synaptic connexions.

It seems certain from Tables 1 and 2 that, when Per motoneurones are transformed to have an 'ankle-extensor' function, there is an increase in the aberrant monosynaptic connexions from the post-tibial extensor muscles to them. Before considering these changes as arising on account of the transformed motoneurone function and being specifically related thereto, it should be considered whether there is an entirely non-specific explanation. Since the cross-union operation resulted in the chromatolytic death of about two thirds of the spinal ganglion cells, their degenerating central terminals may have stimulated sprouting of adjacent Ia afferent fibres which then established the aberrant synaptic connexions on the Per motoneurones. This effect of degenerating nerve fibres was first demonstrated in partial muscle denervation (Hoffman, 1950, 1951; Edds, 1950, 1953), and it has also been found in the partially denervated sympathetic ganglion (Murray & Thompson, 1957) and in the spinal cord when spinal cord section caused degeneration of the descending tracts (McCouch, Austin, Liu & Liu, 1958).

If this non-specific central sprouting caused the increased aberrancy of monosynaptic connexion to Per motoneurones seen in Table 1, it is possible that proximity of the motor nuclei provides a sufficient explanation of the preponderance of the LG aberrant connexions. Each motoneuronal nucleus has a wealth of fine branches of Group Ia primary afferent fibres, particularly from the muscle it innervates; hence motoneuronal nuclei in close proximity are optimally placed for the operation of any influence that degenerating Ia afferent fibres in one may have in causing the sprouting of Ia fibres in the other. Thus LG motoneurones are as a rule closer to Per motoneurones than are the Pl and FDL motoneurones; and degeneration of central terminals has not been associated with the development of aberrant connexions when the respective nuclei were more remote, e.g. from DP nerve to MG motoneurones in Table 1.

It seems unlikely for several reasons that the observed increase in aberrancy can be fully explained by this postulated process of non-specific sprouting. First, the Ia fibres most exposed to the postulated influence would be those actually in the Per nucleus, i.e. the surviving Per fibres and those DP fibres which evoke the heteronymous EPSP (cf. Table 2 and Eccles *et al.* 1957*b*). The mean EPSP of the latter was increased (from 0·94 to 1·50 mV), while with the former there was also possibly a small increase, because chromatolytic destruction reduced the mean Per EPSP on DP motoneurones to 24 %, and on Per motoneurones to 31 %. Thus under optimal conditions non-specific sprouting appears to have increased only to a small extent (by about one third) the EPSPs produced by DP and Per afferents on Per motoneurones, which suggests that it could make but a negligible contribution to the much larger relative increases (over tenfold)

in the EPSPs evoked in Per motoneurones by nerves to the post-tibial extensors (Table 1). Secondly, non-specific sprouting would be expected to increase the aberrant connexions from BST and SM Ia afferent fibres to Per motoneurones, for in the L7 segment their motor nuclei are at least as close to the Per motor nuclei as the FDL nucleus is, yet the observed aberrancy rates in Table 1 are negligible, 2:101 and 0:91 respectively. Thirdly, non-specific sprouting would be expected to increase the aberrancy of monosynaptic connexions not only to Per motoneurones, but also to MG motoneurones—e.g. from the contiguous BST motoneurones—and this has not been observed (Table 1).

There are two further experimental investigations of the postulated effect of degenerating primary afferent fibres in causing sprouting of adjacent fibres. It would be expected that the central degeneration caused by partial severing of the dorsal roots central to the spinal ganglia would cause a large development of aberrant monosynaptic connexions to motoneurones. This possibility is at present being fully investigated. In a preliminary investigation the dorsal root operation was performed on two adult cats and 25 weeks later there were no aberrant monosynaptic connexions on the 96 motoneurones from which intracellular records were made. In the alternative control experiment the Per and MG nerves have been severed, as in the cross-union operation, but have been rejoined to their own peripheral stumps. Under such conditions there will be the same chromatolytic destruction of spinal ganglion cells as in the cross-union operation, but no change in motoneurone function. Comparison of the aberrancies under such conditions with those of Table 1 should establish the degree to which non-specific sprouting is responsible for the aberrancies shown for Per motoneurones in Table 1. Operations of this type have been performed on young kittens, but some months must elapse before the final experimental tests, which will be separately reported.

Following a comprehensive investigation of the receptive fields for the monosynaptic activation of many different species of motoneurones (Eccles *et al.* 1957*b*), the following question was raised, but not answered. What developmental processes are responsible for establishing the highly selective connexions which have been shown to exist? For example, the nuclei of BST motoneurones on the one hand and of the MG and LG motoneurones on the other are in close apposition, yet their respective Ia receptive fields are usually quite independent. Still more remarkable are the connexions made between widely separated but functionally related nuclei, the best instance being the reciprocal gracilis-semitendinosus linkage (Eccles *et al.* 1957*b*; R. M. Eccles & Lundberg, 1958), which occurs despite the location of the respective nuclei at L5 and L7 segmental levels. It does not seem possible to postulate that these highly specific

intersegmental connexions are established by the ingrowth of fibres from the primitive spinal ganglia along the easiest channels between tissue planes. On such an explanation aberrant connexions would be expected to be far more frequent than is actually observed.

One possible explanation of the development of selective connexions is that some specific chemical or physico-chemical property of the Group Ia fibres provides a 'selective contact guidance' during growth; and furthermore, limits the possibility of their functional connexion, so that connexions are made only to those motoneurones that have a complementary specificity (Sperry, 1951*a*; Weiss, 1950). An influence of a muscle on the motoneurones innervating it was postulated by Wiersma (1931) and by Weiss (1936, 1947, 1952). This postulate of a 'modulating' influence of muscle on motoneurones, 'myotypic specification', has been developed by Sperry (1951*a*, *b*; 1958) and Weiss (1952) into the concept that the motoneurone could be conditioned in this way to attract and retain one type of synaptic contact rather than another, there being some kind of chemical affinity of 'specification' between a nerve cell and the synaptic knobs in contact with it. This concept certainly provides a plausible explanation for the development of reciprocal monosynaptic connexions between muscles operating synergically at only one joint; but it encounters difficulty in explaining the central connexions of double-joint muscles, which may receive monosynaptic connexions from the muscles restricted to either of the two joints, and also activate motoneurones which innervate muscles operating either joint (Eccles *et al.* 1957*b*; R. M. Eccles & Lundberg, 1958). The complex monosynaptic linkages between knee and hip flexors and extensors (R. M. Eccles & Lundberg, 1958) provide still greater difficulties for an explanation by specification. Yet, on the other hand, specification as a controlling factor is suggested by the finding that no motoneurone receives both excitation and inhibition from the Group Ia fibres of any particular muscle (R. M. Eccles & Lundberg 1958). The postulated myotypic specification must be distinguished from the chemical transmitter mechanism at synaptic junctions; the former is responsible for the actual existence of the synapses, the latter for their functional operation. Yet it seems very probable that there is a relationship, otherwise the specification could cause the development of synaptic contacts that were functionless because the chemical transmitter was ineffective.

At present the only alternative to the explanation by specification is that initially all varieties of functional connexion are made by the ingrowing Ia fibres, and that this virtual randomness of connexion is gradually shaped to the specific adult patterns by a process of 'resculpturing', by means of which all inappropriate connexions regress, while there is a complementary

enhancement of those synaptic connexions 'corresponding' to proper function (Sperry, 1951*a*).

When considering the experimental results in relation to the alternative hypotheses of 'specification' or 'resculpturing', it seems clear that the former hypothesis alone is supported by the monosynaptic excitatory connexions. The resculpturing hypothesis would predict that there would be a loss of monosynaptic connexions from the control synergic muscles which functioned as antagonists after the cross-union. For example, there should be decreases in the EPSPs produced by DP nerve on the transformed Per motoneurones, and by LG nerve on transformed MG motoneurones, whereas comparison of Tables 1 and 2 shows that there was either a slight increase or no change. On the other hand, the large increase (over tenfold) of the aberrant LG connexions to Per motoneurones can be accounted for by the hypothesis of myotypic specification. It has to be postulated that, as a consequence of the innervation of MG muscle, the Per motoneurones develop a changed surface specificity that attracts LG synapses. However, until the further control experiments evaluate the contribution of non-specific sprouting to the increased aberrancy, the present experiments do not provide convincing evidence for this hypothesis of linked modulation and specification.

The effect of the changed function of Per motoneurones on the incidence of disynaptic Ia inhibition upon them also indicates the existence of some specific factor. In the normal control experiments the Ia IPSP from the antagonist muscles (the post-tibial extensors) was as frequently observed with Per (22:49) as with DP (25:56) motoneurones. With Per motoneurones reinnervating MG muscle there was a large reduction to 9:106 in the incidence of Ia IPSP from the post-tibial extensors, which have been transformed from antagonists to the acquired synergists of the Per motoneurones. In contrast, the Ia IPSPs on the adjacent DP motoneurones were just as frequent (46:107) as normally. There appears to be some incompatibility between Ia inhibitory and excitatory action by any particular nerve on the same motoneurone (cf. R. M. Eccles & Lundberg, 1958). Hence the observed shedding of Ia inhibitory connexions to the transformed Per motoneurones may be a necessary preliminary before Ia excitatory connexions can be established from the acquired synergists. Evidently such suggestions imply that a specific 'modulating' influence from the MG muscle operates on the Per motoneurones reinnervating it, which would be a further example of the process of myotypic specification postulated by Weiss and by Sperry. The apparent shedding of inhibitory synapses is in accord with the resculpturing hypothesis; but these observations must be regarded with caution, unless substantiated by further investigations.

However, even if myotypic specification is responsible for the observed

changes in Table 1, the magnitude of this effect is far less than would be expected for a process that is postulated to account for the embryological development of specific connexions. Possibly specification is much more effective at the very early stage of development. As postulated by Weiss (1947) and Sperry (1951*a*, *b*, 1958), one can envisage the growing nerve fibre as chemically sensing the surfaces along which it grows, and being specifically attracted by the chemical properties of some surfaces and not by others. There are now many lines of evidence which suggest that the development of synaptic connexions can be explained only by some such postulate of surface specificity. For example, Group Ia and Ib primary afferent fibres of muscle appear never to make functional synaptic connexions on the same nerve cell, whether this be a motoneurone, an interneurone of the intermediate nucleus, a cell of Clarke's column, or a cell of origin of the ventral spinocerebellar tract (Oscarsson, 1957; Lundberg & Winsbury, 1960; Eccles, Eccles & Lundberg, 1960). Apparently these two types of fibre differ in respect of the surfaces which attract them. In the regeneration of peripheral nerve fibres, not only do sensory and motor fibres display discrimination in the choice of pathway (Weiss & Edds, 1945; Gutmann, 1945), but there are also discriminations between the various sensory modalities, e.g. between taste buds, lateral-line organs and cutaneous endings (Sperry, 1951*a*). It remains to be determined whether the developments of aberrant monosynaptic connexions here reported are further instances of the influence of specific chemical affinities, i.e. of the postulated specification process, and, in addition, of changes which muscles can produce in motoneuronal specification, the postulated myotypic specification.

SUMMARY

1. In eleven kittens 1–25 days old, nerves to antagonistic leg muscles were cross-united, and some months later intracellular recording of the monosynaptic excitatory post-synaptic potentials was employed to test if any change had been produced in the synaptic connexions to motoneurones with inverted function. The motoneurones and afferent fibres of synergic muscles were employed as controls in the experimental tests.

2. After crossing of the nerve to the peroneal muscles (peroneus longus, brevis and tertius) to medial gastrocnemius muscle, 40 % of the peroneus motoneurones (41:102) were monosynaptically activated from their acquired synergist, lateral gastrocnemius, whereas normally this aberrant connexion was rare (3:52). There was also a large increase in monosynaptic activation from other post-tibial extensors.

3. Correspondingly, there appeared to be a considerable decrease in the inhibitory action exerted by Group Ia afferent volleys from the post-tibial extensor muscles to peroneus motoneurones.

4. No equivalent change occurred in the monosynaptic excitatory action on the medial gastrocnemius motoneurones reinnervating the peroneus muscles.

5. There was a loss of about two thirds of the Group I afferent fibres of the cross-united nerves, presumably on account of chromatolytic death of the spinal ganglion cells.

6. The degeneration of the fine central terminals of these afferent fibres probably caused sprouting of adjacent fibres, so providing a partial explanation of the increased aberrancy of post-tibial extensors to peroneus motoneurones; however this effect appeared to account for only a small fraction of the observed aberrancy.

7. The alternative explanation attributes the aberrancy to a change in peroneus motoneurones consequent on their reinnervation of medial gastrocnemius muscle, whereby they attract synaptic endings from the Ia afferent fibres from their acquired synergists (the process of myotypic specification of Weiss and Sperry). It is hoped that further experimental investigations will allow a relative evaluation of these alternatives.

8. Brief consideration was given to the relation of these findings to the factors responsible for embryonic development of specific connexions in the central nervous system.

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TYPES OF NEURONE IN AND AROUND THE INTERMEDIATE NUCLEUS OF THE LUMBOSACRAL CORD

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Certain types of neurone of the spinal cord have been specially investigated, and as a consequence there are sharply defined categories of cell: alpha and gamma motoneurones, Renshaw cells, cells of origin of the dorsal spinocerebellar tract, and of the ventral spinocerebellar tract. This paper is devoted to an attempt to define additional types of neurone, particularly types of interneurone. This definition of cell type has been based on the synaptic connexions impinging on them and on the destination and functions of their axons. These two criteria will be employed in the classification attempted here for cells located exclusively in and around the intermediate nucleus at L6, L7 and S1 segmental levels. The designation 'interneurone' will be restricted to neurones whose axons were confined within the lumbosacral cord. It is particularly important to restrict the criterion of synaptic connexions to monosynaptic connexions either from primary afferent fibres or from other identifiable fibres which can be directly stimulated. When interneurones are interpolated on the afferent pathway to the cell under investigation, criteria of specificity no longer obtain, because the serial arrangement of only one or two interneurones causes a very wide spatial and temporal dispersal of the various afferent inputs. For example, Group III afferent fibres from muscles or high-threshold afferent fibres from skin can activate many neurones of the types which are defined here by the criteria of their monosynaptic connexions.

The present classification follows on from a preliminary attempt (Eccles, Fatt, Landgren & Winsbury, 1954; Eccles, Fatt & Landgren, 1956), in which interneurones in the intermediate nucleus were distinguished according to their monosynaptic activation by Group Ia or Group Ib afferent impulses from muscle. Brief reference was also made to a third category of neurone monosynaptically activated by cutaneous afferent impulses. Since that time there has been a very comprehensive attempt at interneuronal classification by Kolmodin (1957), which was based on the receptor pathways activating interneurones. Unfortunately,

activation by adequate stimulation precludes accurate latency measurements, which are essential in defining monosynaptic connexions; hence Kolmodin's classification is complicated to an unknown degree by activation through polysynaptic channels. In addition, no attempt was made to discriminate between the different stretch receptors of muscles, e.g. between annulospiral endings, Golgi tendon organs and secondary endings in muscle spindles. It is, therefore, not possible to attempt any synthesis between Kolmodin's classification and the partial classification attempted here. All the types defined by Kolmodin (1957) were also widely dispersed throughout the grey matter.

Altogether almost 100 neurones have been arranged in three main types; but with about thirty others this has not been possible, either because the data were inadequate or because they belonged to types not yet defined. Finally, the most common type of interneurone has eluded our classification; such interneurones occur ubiquitously throughout the grey matter, and are activated to fire repetitively by high-threshold afferent fibres in both muscle and cutaneous nerves.

There is now a considerable literature on the electrophysiological properties of interneurones as studied by intracellular recording (Woodbury & Patton, 1952; Frank & Fuortes, 1955, 1956*a*; Suda, Koizumi & Brooks, 1958; Haapanen, Kolmodin & Skoglund, 1958; Kolmodin & Skoglund, 1958; Hunt & Kuno, 1959*a*, *b*; Wall, 1959; Kostyuk, 1959). It will emerge that, so far as the interneurones themselves are concerned, the *A* and *B* types defined here are not distinguishable by their electrophysiological properties. On the other hand, the cells of origin of the ipsilateral cutaneous tract (*CT* type) resembled motoneurones in many respects; the spike showed a similar initial segment–soma dendritic (IS–SD) composition and was followed by a long after-hyperpolarization (100 msec). These tract cells were also much more tolerant of intracellular electrodes than were interneurones.

METHODS

The experiments were performed at the same time as the investigations reported in three previous papers (Eccles, Eccles & Lundberg, 1957*a*, *b*, *c*), and the methods of experiment were identical with those described there. The micro-electrodes were filled with 3 M-KCl and usually had a resistance of 5–10 MΩ. A wide range of nerves were mounted for stimulation and could be tested in quick succession in the way previously described. Special precautions were taken in freeing the nerves so that their blood supply was preserved; and they were mounted on electrodes with an interpolar distance of about 1·5 cm so that the stimulating current would be uniformly applied to the nerve fibres, and there would be a negligible influence of the killed and at the region of application of the cathode. The nerve volleys evoked by the stimuli were always monitored by a surface-recording electrode applied to the spinal cord at the point of entry of L 7 dorsal root, and recorded against the large indifferent earth electrode, which was applied to the back muscles. No potential scales are given, in the figures, for these records, which were employed to reveal the Ia, Ib composition

of the ingoing volley and also to time the entry of the Ia and Ib volleys into the spinal cord. Throughout this paper the entry has been assumed to occur at the first reversal point of the triphasic surface records of the Ia and Ib afferent volleys. In most experiments the spinal cord was severed at the lower thoracic level and the ventrolateral columns on each side were prepared and mounted on stimulating electrodes (Laporte, Lundberg & Oscarsson, 1956*a*). In the remaining experiments the cord was severed at the upper lumbar level and no stimulation of the tracts was attempted.

Usually separation of the Ia and Ib afferent volleys could be detected only with biceps-semitendinosus and quadriceps afferent volleys (cf. Bradley & Eccles, 1953; Eccles *et al.* 1957*a*). However, investigations on the disynaptic Ib action on motoneurones have shown that with other nerves from limb muscles (gastrocnemius-soleus, plantaris, flexor digitorum longus, etc.), the Ib threshold was lower, relative to Ia, than with the nerves from thigh muscles (Eccles *et al.* 1957*a*, *c*). This difference in threshold has also been used to some extent in the present investigation. When in a particular experiment it was observed that one group of interneurones was excited by low-threshold Group I afferents and another group by high-threshold Group I afferents, it was assumed that these afferents were Ia and Ib, respectively. By recording from motoneurones in the same experiments it was often possible to verify this assumption when Ia synaptic actions from the particular nerves were evoked exclusively at low strength and Ib action exclusively at higher Group I strength. Ib actions were seldom observed with a stimulus below 1·2 times threshold for Group I fibres, and as tested by the monosynaptic excitatory post-synaptic potential (EPSP) maximum Ia volleys were usually generated by stimuli only 1·25–1·35 times threshold (cf. Eccles *et al.* 1957*a*, *c*).

The micro-electrode was always inserted from the dorsum of the cord medial to the line of root entry, the direction and depth of the tracks being in general similar to those plotted by Eccles *et al.* (1954). When the recording was intracellular, records were usually taken on withdrawal of the electrode from the cell in order to determine the field potential. These records have not been reproduced, since the external fields were negligible relative to the large intracellular potentials. Many of the spike potentials in the figures have been retouched.

RESULTS

Monosynaptic action by Group Ia fibres of muscle (type A)

Altogether there have been 27 interneurones monosynaptically activated by Group Ia fibres of one or more muscle nerves. They have been located in the zone of synaptic activation by Group Ia volleys from muscle, as indicated by the focal synaptic potential (Eccles *et al.* 1954, Figs. 2, 4), which approximately coincided with the intermediate nucleus of Cajal (1909). The most superficial was at 1·95 mm and the deepest at 3·3 mm from the dorsum of the cord; i.e. they were all dorsal to the motoneuronal nuclei. None of the fourteen cells so tested was antidromically activated from the spinal funiculi of either side, though many were synaptically activated by descending volleys (Fig. 1M). On account of their similarity in other respects, monosynaptic Ia activation and location, it was assumed also that the fourteen untested cells did not have their axons in the ascending tracts of the cord. As a rule type *A* interneurones were rapidly killed by a penetrating micro-electrode, so most have been studied by extracellular recording. Nevertheless, five have been extensively investi-

gated intracellularly. Their membrane potentials ranged from −50 to −70 mV.

The potentials of Fig. 1A–F were evoked by graded stimulation of the biceps-semitendinosus nerve at strengths from just above threshold for Group Ia (A) to supramaximal for Ib (F). There was a very high level of background synaptic noise, which is characteristic of most types of interneurone. Superimposed thereon were excitatory post-synaptic potentials

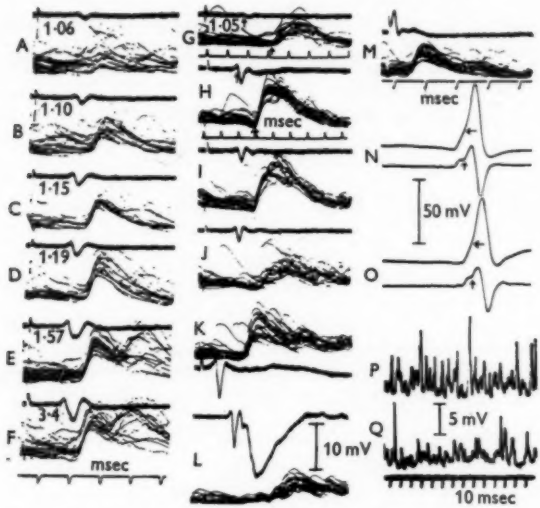

Fig. 1. Intracellular recording from a type *A* interneurone in the intermediate nucleus at a depth of 2·4 mm from the cord dorsum and in the middle of L 7 segment. The membrane potential was about −60 mV throughout. The high background of synaptic noise (P) was diminished (Q) after injection of pentobarbital sodium 5 mg/kg. A–F, superimposed traces of EPSPs evoked by stimuli of progressively increasing strength applied to biceps-semitendinosus nerve, the numbers giving strengths relative to the threshold stimulus. The potential produced by the afferent volley in the L 7 dorsal root (surface recording) is shown above the corresponding intracellular record. Upward deflexions in both signal increasing positivity relative to the indifferent earth electrode. G and H, similar recording of potentials evoked by a very weak (1·05T) and a maximum Group I stimulus to gastrocnemius nerve, Ga; I–K, similar recording for maximum Group I volleys for plantaris, Pl; flexor digitorum longus, FDL; quadriceps, Qu, nerves. L and N similarly recorded, but for volleys evoked by stimuli of several times threshold strength in superficial peroneal (SP) and the ipsilateral ventrolateral funiculus (IF). N and O are spikes arising from EPSPs set up by Pl and Ga volleys (cf. I, H) when the recording conditions had improved so that the larger EPSPs generated spikes at the arrows. Lower traces in N and O are the electrically differentiated records, showing abrupt transition from EPSP to spike at the arrows. Various time and potential scales are shown at appropriate places, A–F having the same time scale, as also H–M. Note that G was faster than H. Same potential scale for A–M.

even with the smallest Group Ia volley (seen best at the lower margin of the superimposed traces). The latent period was about 0·35 msec from the time of arrival of the afferent volley at the spinal cord. When there was a Ib afferent volley in addition, there was often a later EPSP (E, F) with a latency of about 1·25 msec, measured from the time of arrival of the Ib volley. This indicates the interpolation of an interneurone on the Ib pathway (cf. Eccles *et al.* 1957*c*).

Potentials generated by afferent volleys from other muscle nerves, and by a descending volley in the ventrolateral funiculus of that side, are shown in Fig. 1G–M. By graded stimulation it was shown that the lowest threshold afferent fibres of both the gastrocnemius and plantaris nerves were effective in generating the earliest EPSPs of Fig. 1H and I, respectively. For example, the stimulus to gastrocnemius nerve was at a strength only 5% above threshold in G. Since the central latency of the EPSP in H and I was 0·6–0·7 msec longer than for the BST response, it is likely that an interneurone was interposed on these Ia excitatory pathways.

The time course of the monosynaptic EPSP's generated by the Ia afferent volleys is better illustrated in Fig. 2A–D, where the background of synaptic noise was much lower. Just as with motoneurones, increasing the size of the afferent volley increased the EPSP without greatly altering its time course. The only variant consisted of the superposition of a small partial spike response on the largest EPSP (D). The critical level at which an EPSP generated the spike is shown in G and H, though a partial spike response was superimposed on the EPSP in F. The latent period for the EPSP was 0·6 msec with the just threshold condition obtaining in E and F.

The potentials following the spike are well shown when two traces are superposed, as in Fig. 2K, but they are also seen by comparing E and F with G and H. The spike rapidly declined to a brief after-hyperpolarization, just as observed with motoneurones having low membrane potentials (Coombs, Eccles & Fatt, 1955); but there was, in addition, a much more prolonged after-hyperpolarization, which, in K, was still large 15 msec after the end of the spike. This long after-hyperpolarization has been seen with other type *A* cells, but has not been investigated at slow sweep speeds to see if it is comparable in duration with the after-hyperpolarization of alpha motoneurones (Eccles, Eccles & Lundberg, 1958). It was certainly more than 50 msec in duration for the interneurone of Fig. 1.

The spikes of Fig. 2 differed from those of motoneurones in that there was no sign of a separation into IS and SD components even with electrical differentiation of very fast sweeps (I, J). The initial slowly rising phase of the EPSP changed smoothly to the steep rise of the spike without any trace of an inflexion, as may be seen also with the spike arising (at the arrow) from the steep EPSP in the undifferentiated records of Fig. 1N, O.

In view of the depolarized condition of cells penetrated by the microelectrodes, the threshold depolarization for initiation of a spike by the EPSP was high, just as with the soma-dendritic membrane of motoneurones (Araki & Otani, 1955; Fuortes, Frank & Becker, 1957; Coombs,

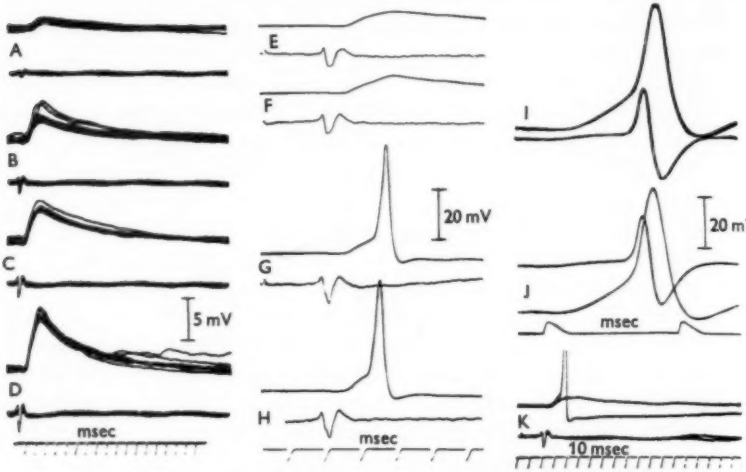

Fig. 2. Intracellular recording from a type *A* interneurone in the L7 segmental level at a depth of 2·0 mm from the cord dorsum. Membrane potential, about −70 mV throughout. A–D are superimposed traces of EPSPs (above) and dorsal root action potentials (below) evoked by progressively increasing stimuli, applied to the combined FDL–Pl nerves. E–H are single traces at faster sweep and lower amplification, the evoking stimuli being about same strength as in C for E and F, and in D for G and H. Lower trace of I and upper of J are electrically differentiated records of the EPSP and superimposed spike evoked by a volley from the posterior tibial nerve, which supplies flexor digitorum brevis and the various toe muscles, there being two superimposed traces in I. K shows, at slow sweep speed, a record of potentials evoked in response to a stimulus of same strength as in C, but at lower amplification. The EPSP was just at threshold, evoking a spike in one of the two traces. Potential and time scales are given at appropriate places, one of the potential scales obtaining for A–D and the other for E–K, as shown. Note very fast sweep for I and J.

Curtis & Eccles, 1957*b*), being about 15 mV in Fig. 1N, O, and 13 mV in Fig. 2I, J. This absence of an IS–SD separation has previously been reported for interneurones (Haapanen *et al.* 1958; Hunt & Kuno, 1959*a*), so it appears that many interneurones lack the zone of low-threshold membrane which occurs in the initial segment of motoneurones.

The interneurone of Fig. 2 was excited through polysynaptic pathways by cutaneous afferent fibres. As is indicated by the EPSPs of Fig. 3, single volleys in cutaneous nerves produced a prolonged irregular bombardment,

particularly when set up by strong stimuli (D, H). These EPSPs generated spike potentials (Fig. 3J, K) if they attained the threshold level of depolarization (about 8 mV). The interneurone of Fig. 1 was also excited by a polysynaptic cutaneous path (L).

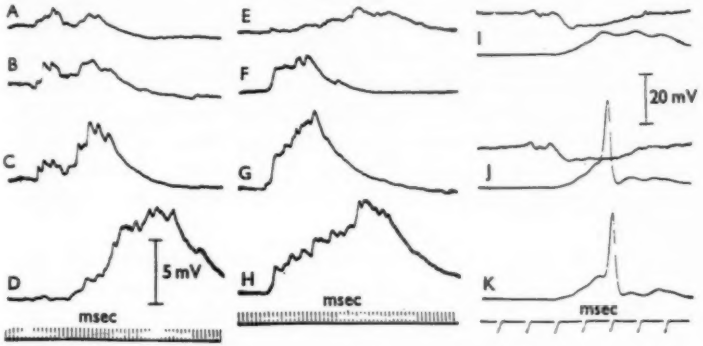

Fig. 3. Intracellular recording from same interneurone as in Fig. 2, A–D and E–H showing prolonged irregular EPSPs produced by stimuli of progressively increasing strength to two cutaneous nerves: sural, A–D, and superficial peroneal, E–H. I–K show records, at lower amplification and faster time base, of potentials evoked by a superficial peroneal volley, the EPSPs evoking a spike in J, K. Upper traces in I and J are potentials led from the L7 dorsal root at its cord entry. Upward deflexion always signals increasing positivity relative to earth. Potential and time scales are given at appropriate places.

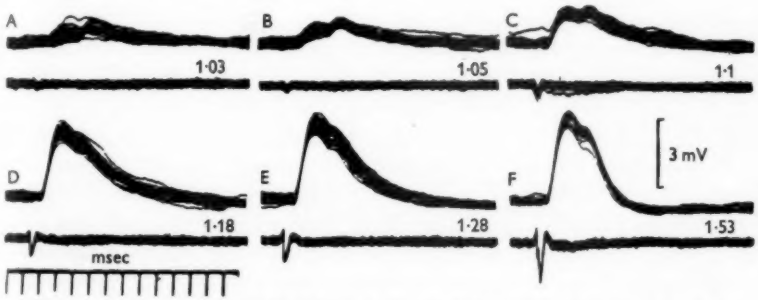

Fig. 4. Intracellular recording from a flexor digitorum longus motoneurone (upper trace) on stimulation of the FDL nerve at the indicated strengths relative to threshold. The lower traces are from the L7 dorsal root entry zone, negativity being signalled downwards, and show that the Group I volley displayed the separation in Ia and Ib components.

It was unexpected to find (see Fig. 1G–I) that Group Ia impulses generated an EPSP after such a long latent period that it was necessary to postulate an interpolated interneurone. It is relevant to report here

that there is some evidence of Ia excitatory action occasionally reaching ipsilateral motoneurones by a polysynaptic pathway. In the experiments of Fig. 4 the nerve to flexor digitorum longus displayed separation in Ia and Ib components (D–F). A single volley from this nerve evoked in a flexor digitorum longus motoneurone a double EPSP which is best seen in records A–C, Fig. 4. The central latent periods were 0·6 and 2·6 msec which would be monosynaptic and probably trisynaptic respectively. High-threshold Group Ib fibres evoked the usual IPSP in F. The late EPSP component was evoked in A at a strength 1·03 times threshold for the nerve, and this was also the threshold strength for the fibres with monosynaptic connexions to the motoneurone. Hence it is difficult to escape the conclusion that the polysynaptic EPSP in Fig. 4 was evoked by impulses in Ia afferents.

None of this series of 27 interneurones has exhibited monosynaptic activation by Ib as well as by Ia impulses of the same or another nerve. Thus there was no overlap between type *A* and type *B* interneurones. However, type *A* interneurones were sometimes disynaptically activated by Ib impulses, as illustrated in Fig. 1A–F.

Extracellular records of type *A* interneurones have been shown previously (Eccles *et al.* 1956, Figs. 2, 4, 5). Since only spike potentials can be recognized, the information on synaptic activation was limited, though specification by monosynaptic Group Ia activation was essential before the interneurone could be classed as type *A*. It is conceivable that some of the interneurones that could not then be classified were subliminally activated by Group Ia volleys.

Many of the type *A* interneurones were very restricted in their synaptic activation, as illustrated in Fig. 5, where the cell was effectively activated by very low-threshold Group Ia volleys from biceps-semitendinosus (A), but by no other afferent volley from muscle or skin. Tests by the double-volley technique (F–I, cf. Fig. 2, Eccles *et al.* 1956) showed that maximum Group Ib volleys (H, I) did not excite a discharge from this cell. However, the cell was synaptically excited from the ventrolateral funiculus with a latency of 2 msec after the arrival of the descending volley, which would suggest a pathway through one or two interneurones. The power of the monosynaptic activation from biceps-semitendinosus was shown by the responses to repetitive stimulation, the cell following perfectly all tested frequencies, even as high as 480/sec (L).

In contrast, some type *A* cells discharged impulses in response to a wide range of high-threshold afferent fibres, as has already been reported (Eccles *et al.* 1956, Fig. 5). A very wide field of action is shown in Fig. 6 for an interneurone which was activated monosynaptically only by Group Ia fibres from biceps-semitendinosus. This activation was powerful; fast

records gave a central latency of only 0·5 msec for the spike (cf. first spike of G), and there was very good following of a stimulus frequency of 680/sec (I). The response to strong stimulation of the muscle and cutaneous nerves was a repetitive discharge beginning after a long latency for all but flexor longus digitorum (D), and this exceptional discharge was probably spontaneous (cf. H), for it was not seen with weaker stimulation of this

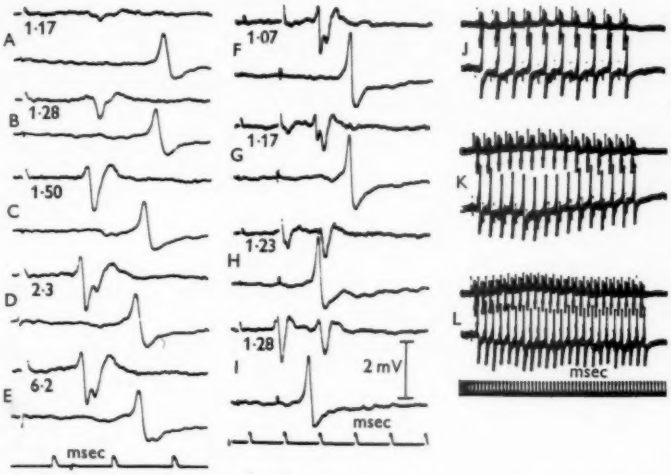

Fig. 5. Extracellular recording from a type *A* interneurone in the intermediate nucleus at a depth of 2·5 mm from the cord dorsum. In A–E the stimuli to BST nerve were at the indicated strengths relative to threshold, the upper trace being recorded as usual from the L7 dorsal root, upward deflexion signalling positivity. In F–I a second stimulus was given at a fixed interval of 1·1 msec after the first and at a strength (2·5T) supramaximal for Group I, while the first stimuli were at the strengths indicated on each trace. In J–L the BST nerve was stimulated at a strength of 6·2T and at frequencies of 230, 345 and 480/sec, respectively. Same potential scale obtains throughout for the micro-electrode records (upward deflexions signalling negativity), and the time scales belong to the appropriate columns. No other afferent nerve nor descending tract fibres had any action on this interneurone.

nerve. These late bursts of discharges were evoked by Group III afferent volleys, which are very powerful in activating interneuronal pathways, so giving prolonged reflex discharges (Lloyd, 1943; Eccles & Lundberg, 1959*b*).

Type *A* cells have usually received monosynaptic Ia innervation from only one muscle nerve. Exceptionally there was double innervation, even from such widely divergent muscle groups as quadriceps and the anterior tibial muscles.

Monosynaptic activation by Group Ib fibres of muscle (type B)

Type *B* interneurones have been specified by two criteria, monosynaptic activation by Group Ib impulses from muscles and absence of antidromic invasion from the ventrolateral funiculi on either side of the spinal cord. This second criterion distinguishes between interneurones and tract cells, but it has been applied with only 21 of the total of the 30 interneurones classified as belonging to type *B*. In addition to these 30 there

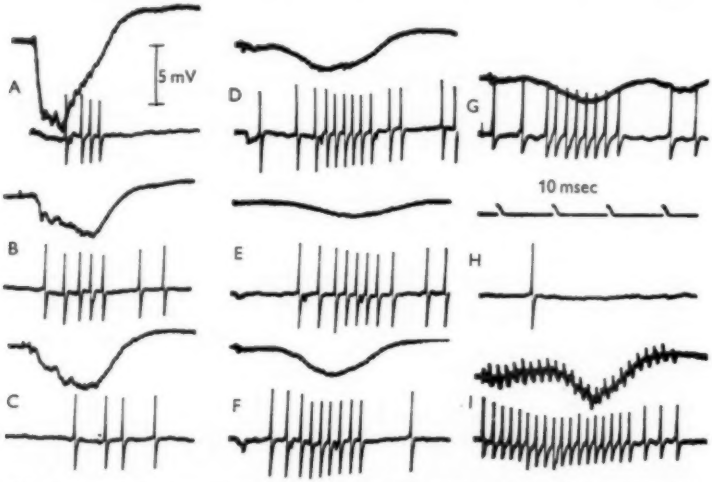

Fig. 6. Lower traces were extracellular recordings from a type *A* interneurone activated monosynaptically only by the Ia fibres of BST, the spike discharge having the brief central latency of 0·55 msec. Upper traces were recorded as usual from the L7 dorsal root entry. A–G are responses evoked by single stimuli at a strength sufficient to excite Group III fibres of muscle nerves and the corresponding fibres of cutaneous nerves: A, SP; B, sural; C, saphenous; D, FDL; E, Pl; F, gastrocnemius; G, biceps-semitendinosus; H shows one of the occasional responses occurring spontaneously; and in I the BST nerve was stimulated at 680/sec, by stimuli supramaximal for Group I.

were four interneurones which were not sufficiently investigated to determine whether their monosynaptic activation was through Ia or Ib fibres, or through both. Apart from this small indeterminate group, type *A* and *B* cells appeared to be sharply distinguishable, none being monosynaptically activated by both Ia and Ib impulses from muscle. Type *B* cells were located at depths ranging from 1·5 to 3·5 mm below the dorsum of the spinal cord, all but two lying between 1·9 and 3·2 mm, which is within the area of extensive Ib synaptic action as revealed by the large extracellular synaptic potentials produced by Ib volleys (Eccles *et al.* 1954).

A rigorous distinction between Ia and Ib afferent volleys was possible when the respective spikes were distinguishable in records led from the appropriate dorsal root, as in the upper records of Fig. 7A–F. Discrimination between the actions of the Ia and Ib volleys was effected by

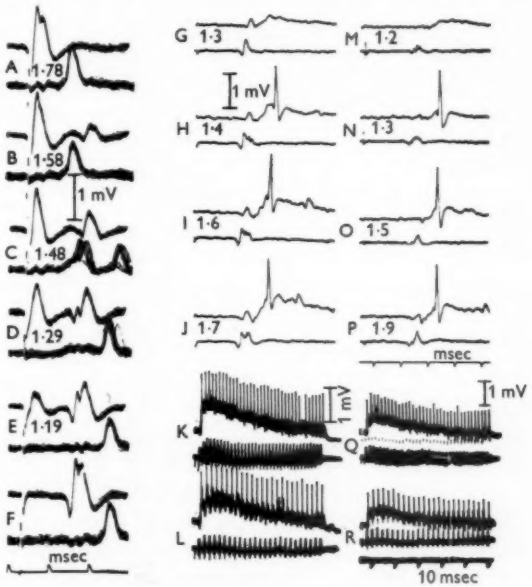

Fig. 7. A–F: Extracellular records of a type *B* interneurone (depth of 3·5 mm) activated from BST nerve, and with double stimulation as in Fig. 5F–I. Second stimulus was at a constant strength, supramaximal for Group I, and at 0·93 msec interval. First stimuli were at the indicated strengths relative to threshold. In both the upper dorsal root record and the lower micro-electrode record, upward deflexions signal negativity, but potential scale is only for micro-electrode traces. All records were made by superposition of about forty faint traces. G–R: Extracellular records of a type *B* interneurone at a depth of 2·4 mm in another experiment. It was activated both by gastrocnemius (G–L) and plantaris (M–R) afferent volleys. The upper trace was always from the micro-electrode (same potential scale throughout except for Q and R), the lower from the lower L7 dorsal root entry. The strengths of the single stimuli are given relative to the threshold of that nerve. The repetitive stimuli were well above maximal for Group I and were at 670/sec (K, Q) and 460/sec (L, R).

varying the strength of the stimulus applied to the biceps-semitendinosus nerve at 1·15 msec before a second testing stimulus, which was at a constant strength supramaximal for Group I (F). As is shown in A, the testing stimulus was unable to excite any Group I fibres that had been excited by the first stimulus. In B the first stimulus (1·58T) was submaximal for Ib.

C shows a transitional state where the first stimulus (1·48T) excited only about one third of Group Ib, as indicated by the large Ib spike set up by the second stimulus; yet it still evoked the interneuronal spike on every test, though with a longer and more variable latency than in A and B; but now the Group Ib volley set up by the second stimulus was also effective, so that the interneurone usually discharged twice at an interval of about 1 msec. The next weakest initial stimulus (1·29T), in D, excited very few Group Ib fibres and never evoked a discharge of the interneurone, though it was almost maximal for Group Ia, as revealed by the small Group Ia spike set up by the second stimulus. E shows a further stage of the series (first stimulus 1·19T) where the second stimulus excited about half of Group Ia and probably all of the Ib fibres. In contrast to C, and even D, it was now just as effective in exciting the interneurone as the maximal Group Ia–Ib volleys in A and F. It may be concluded that the interneurone was very effectively excited by Group Ib impulses, and that there was no evidence for a Ia excitatory action. The incomplete separation between Ia and Ib prevented such analyses from being decisive (cf. Laporte & Bessou, 1957; Eccles *et al.* 1957*a*), and it is desirable to have tests by adequate stimulation of receptor organs (cf. Lundberg & Winsbury, 1960*a*, *b*).

The series of Fig. 7G–R shows extracellular records of another interneurone classified as type *B* by graded stimulation of nerves to gastrocnemius (G–J) and plantaris (M–P) muscles. The gastrocnemius afferent volley showed a separation into Ia and Ib spikes (G–J). In G, where the Ib volley was small or absent, the interneurone was not excited despite the almost maximal size of the Ia volley. Figure 7M–P shows that the interneurone was also excited by the higher threshold component of the Group I plantaris volley, which presumably was also a Ib action (cf. Eccles *et al.* 1957*a*). The generation of impulses in the interneurone was accomplished in the short central delay of 0·7–0·8 msec for both plantaris and gastrocnemius afferent volleys. An intense excitatory action of both afferents was further shown by the responses to repetitive stimulation, there being virtually a perfect following of frequencies as high as 670/sec (Fig. 7K, L, Q, R). This type *B* cell was also excited by superficial peroneal volleys after a long central delay, but by no other muscle afferents.

There was a much more extensive receptive field for the type *B* cell of Fig. 8, though the frequency tests showed that, except for the plantaris afferent volley (D, E), it was a field of low intensity (I, J, N, O). The plantaris volley also showed its effectiveness in the very short central latency (0·6 msec in C) and relatively small size of the Group I volley (A) which evoked a discharge from the interneurone. By contrast, the gastrocnemius Group I volley had to be almost maximal (F) and the minimum

central latency was 0·9 msec (H). It was of interest that, although the Group I volley from flexor digitorum longus had to be almost maximal in order to be effective (K–M), it evoked a double discharge even at threshold, the central latencies for the two discharges being about 1·0 and 2·0 msec with a maximal Group I volley. Three criteria established that the

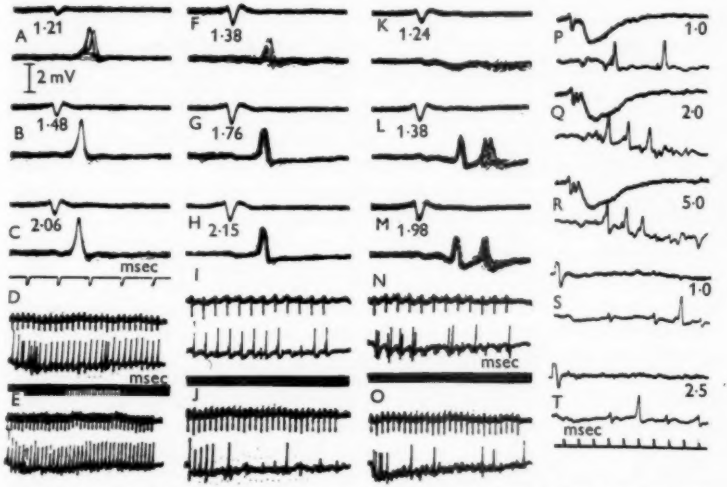

Fig. 8. Extracellular records from a type *B* interneurone in the intermediate nucleus at a depth of 2·1 mm from the cord dorsum. Upper traces are potentials led from the L 7 dorsal root entry, positivity upwards. Same potential scale for all micro-electrode records, upward deflexions signalling negativity. A–E, F–J and K–O are series with stimuli at the strengths indicated applied to plantaris, gastrocnemius and flexor digitorum longus nerves, respectively. In A–C, F–H and K–M the records are formed by the superposition of about forty faint traces. The stimulus frequencies in D, E are 480 and 690/sec, while in I, J and N, O they are 220 and 480/sec, respectively. In P–R single stimuli were applied to the superficial peroneal nerve at the strengths indicated relative to that of P, and similarly in S and T to the quadriceps nerve. Note different time scales in appropriate places.

Group I b and not the I a impulses from plantaris were effective in exciting the interneurone. The very short central latency of the discharge evoked by a plantaris volley (C) and particularly the very high frequency of repetitive discharge (690/sec followed perfectly in E) showed that a maximal Group I plantaris volley excited the interneurone very powerfully. The tests (A–C) could indicate that the interneurone was weakly excited by I a impulses, the volley evoked at a strength of 1·21 T being just sufficient to evoke a discharge (A); but this interpretation is inconsistent with the powerful excitation by high-threshold Group I afferents revealed by the other tests. Thus excitation by lowest threshold Group I b fibres may be presumed in

Fig. 8A, particularly as a stimulus of strength 1·21T has been found to evoke a disynaptic Ib inhibitory action in motoneurones (Eccles *et al.* 1957*c*, Figs. 3, 4). On this interpretation the interneurone would be excited monosynaptically by Ib not only from gastrocnemius (F–H) and flexor digitorum longus (K–M), but also from plantaris (A–C). In addition,

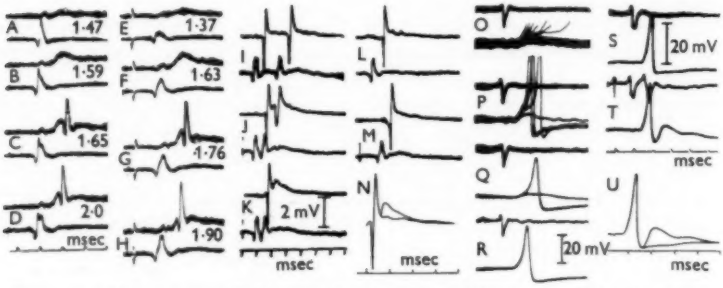

Fig. 9. A–M are extracellular records of a type *B* interneurone in the intermediate nucleus at 2·2 mm below cord dorsum. Upper traces are micro-electrode records (note potential scale), while lower records are from L 7 dorsal root entry, upward deflexions for both records signalling negativity. In A–H records are formed by superposition of about forty faint traces. A–D and E–H are evoked by single stimuli to the gastrocnemius and plantaris nerve respectively at the indicated strengths relative to threshold. In I–K the gastrocnemius stimulus was applied at short intervals before the plantaris stimulus, both stimuli being supramaximal for Group I, and L, M show the respective single responses. Superposition of responses L and K (in N) reveals the time course of the EPSP set up by the plantaris nerve when refractoriness prevented generation of a spike. Actually the tracings of N are the means of two closely similar records. The same interneurone was later penetrated by the micro-electrode, the membrane potential having the low value of −38 mV. O–R show intracellular responses evoked by gastrocnemius afferent volleys set up by a stimulus of 1·59T strength (cf. B), with many superimposed in O, 4 in P, 2 in Q and 1 in R. Note that Q and R are at lower amplification than O, P, S, T. S, T and U are intracellular records corresponding to the extracellular series L, K, N, respectively. Upward deflexions in intracellular records signal positivity.

it was excited at longer latency by a wide range of cutaneous afferents in the superficial peroneal nerve (P–R) and very slightly by high-threshold quadriceps afferents (S, T). Several type *B* interneurones were synaptically excited by descending volleys in the ipsilateral funiculus.

Intracellular records were made from only three of the total of 30 type *B* interneurones. The cell illustrated in Fig. 9 was shown to be type *B* by the threshold tests with gastrocnemius (A–D) and plantaris-flexor digitorum longus volleys (E–H), and was later penetrated by the micro-electrode. A just-threshold gastrocnemius volley then gave the responses (O, P), showing the spike arising from an EPSP at a rather low threshold level

(about 6 mV), which may be attributed to the depolarized state of the cell, the membrane potential being only −38 mV. The spike showed no sign of IS–SD composition and initially had a duration of 0·5 msec (P), but later it lengthened to 0·8 msec (Q, R), there being a large after-hyperpolarization, which seemed to be comparable with that given by type *A* interneurones (Fig. 2K). The large size of the EPSP produced by a maximal Ib volley is shown in T, where a plantaris volley 0·8 msec after a gastrocnemius volley generated only an EPSP, which is revealed by comparison with the control records (S) and in the superimposed tracings (U). During the earlier extracellular series a similar test had been made (K, L, M), showing the time course of the extracellular EPSP in the superimposed tracings (N). At longer volley intervals (I, J) the plantaris volley also evoked a spike in the interneurone, the interval between two successive discharges being as brief as 0·7 msec when the volley interval was 0·5 msec (J). The refractory period of the interneurone was thus very brief and the potency of the synaptic stimulation very high.

Monosynaptic action by impulses from cutaneous nerves (type C)

The interneurones of type *C* are specially concerned in the relaying of impulses from cutaneous nerves, and are monosynaptically excited by impulses from one or another cutaneous nerve. One subgroup has been characterized in addition by being invaded antidromically by impulses descending the ventrolateral funiculus on the same side, i.e. they were tract cells relaying cutaneous impulses up the ipsilateral side. At least 14 of the total of 33 type *C* cells were in this subgroup, which may be called *CT*, and 6 type *C* cells did not have a tract axon, being therefore primarily cutaneous interneurones, and forming a subgroup that may be called *CN*. The remaining 13 type *C* cells were not tested for antidromic invasion from the ventrolateral funiculi. The *CT* cells were remarkable in being much more tolerant of intracellular electrodes than interneurones, and extensive intracellular investigations were possible with four of them.

The cell illustrated in Fig. 10 had a membrane potential of −60 mV throughout a long investigation, and A–D show the responses evoked by progressively increasing stimuli to the superficial peroneal nerve. In A the spike is seen to arise from an EPSP, but with B–D the EPSP was so steep that there was barely any inflexion between it and the initial spike. In C the high spike frequency was associated with depression of size and this effect was even greater with the second and third spikes of D, where the initial frequency was about 1000/sec. The spike potential generated by antidromic invasion from the ipsilateral tract (E) clearly exhibited an IS–SD composition, particularly in the differentiated record. The total

duration of the spike was about 0·9 msec, and it was followed by the brief after-hyperpolarization characteristic of depolarized neurones. Slower records (F, G) revealed a much larger and longer after-hyperpolarization, which was over 100 msec in duration (H), and comparable with that occurring in motoneurones (Eccles *et al.* 1958). Similar after-hyperpolarizations were given by other *CT* cells. Finally, in I–L there was a

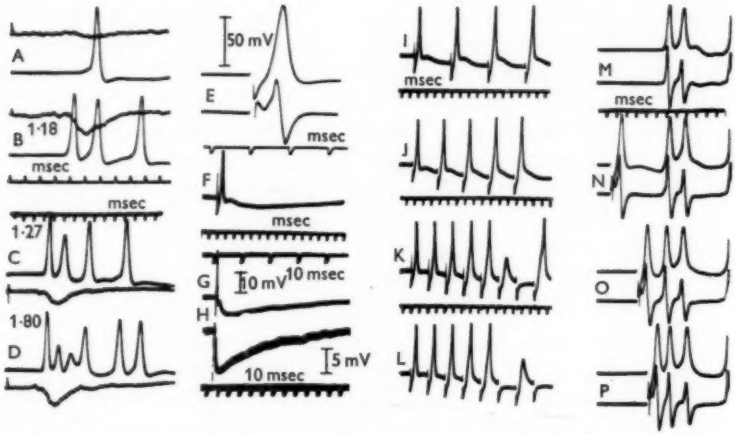

Fig. 10. Intracellular records from a type *CT* cell at a depth of 1·5 mm from the cord dorsum, the membrane potential being −60 mV. A–D are responses evoked by a stimulus to superficial peroneal nerve at the indicated strengths relative to the stimulus for A. Note change in time scale between B and C. There is a record from L 7 dorsal root entry (lower trace) as well as the intracellular record. Upward deflexions of both traces signal positivity. E–H are antidromically evoked potentials in response to a stimulus to the ventrolateral funiculus of the same side, E being an electrically differentiated record. Note changes in the potential and time scales. I–L show responses to antidromic tetani at respectively 230, 340, 490, 490/sec. M shows response to a superficial peroneal volley set up by a stimulus 1·2 times the strength for response, A. In N–P it was preceded at progressively shorter intervals by an antidromic spike response. No other nerves were effective in exciting this cell. Same potential scale for all records except G and H. Note time scales in appropriate places.

repetitive antidromic invasion of the cell, even for 5 impulses at 490/sec; thereafter blockage occurred, much as with motoneurones, for in K and L there were either IS spikes, or no detectable spike. Presumably this was due to an axonal block, just as occurs with motoneurones (Brock, Coombs & Eccles, 1953). The brief refractoriness of *CT* cells is further illustrated by the effect of a preceding antidromic volley on a synaptically evoked response (N–P), the control being shown in M. In P the synaptic stimulation generated a spike at 1·2 msec after the onset of the antidromic spike.

Somewhat similar observations have been made by Wall (1959) on repetitively discharging cells.

Further details of the spike responses of *CT* cells are illustrated in Fig. 11. With the antidromic spike in A, B, there was a separation into IS and SD components equivalent to that occurring in motoneurones (Araki & Otani, 1955; Fatt, 1957; Fuortes *et al.* 1957; Coombs, Curtis & Eccles, 1957*a*). This IS–SD separation also occurred with spikes generated by synaptic excitatory action. With a very small superficial peroneal volley

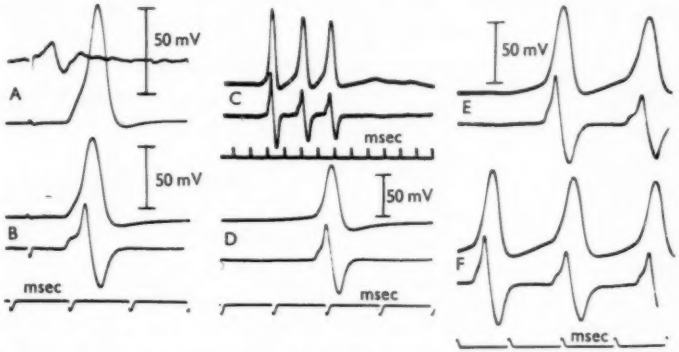

Fig. 11. Intracellular records from a type *CT* cell as in Fig. 10, but in a different experiment. Depth below dorsum of cord was 1·8 mm; membrane potential was not recorded. A gives antidromic spike response to a descending volley in the ventrolateral columns of the same side, the upper trace showing the field generated by the volley. In B the lower trace shows the electrically differentiated record of the antidromic spike. C–F are responses evoked by a superficial peroneal volley together with the electrically differentiated records. D shows response to a volley which just evoked a single spike. D–F have fast time bases and the initial rising phase of the EPSP was not fully recorded in F. Superficial peroneal was the only nerve evoking any response of this cell. Note potential and time scales related to appropriate records, C being at same amplification as E and F.

a single spike was produced by a slowly rising EPSP (D), there being a clear IS–SD composition in the differentiated record, as may also be seen in the repetitive spike discharges evoked by larger afferent volleys (C, E, F). Thus, cells of type *CT* give spike responses and after-hyperpolarizations resembling those of motoneurones; but they differ in that they can discharge at a much higher frequency.

It was remarkable that all the *CT* cells which were fully categorized (13) were activated monosynaptically by the superficial peroneal nerve, and none by the other cutaneous nerve (sural), which was always tested. These *CT* cells were, however, often activated by high-threshold muscle afferents, giving a repetitive response after a long latent period.

Figure 12 illustrates the responses of a cell belonging to the other subgroup (type *CN*). Such cells were often synaptically activated by descending impulses in the ventrolateral funiculus of the same side, as shown by the various sizes of EPSPs evoked by graded volleys (A–C). In G and H the EPSP evoked a spike after a rather long rising phase, and differentiation (I) showed that it also had an IS–SD composition. Finally, in D–F are spike responses evoked by progressively increasing volleys in

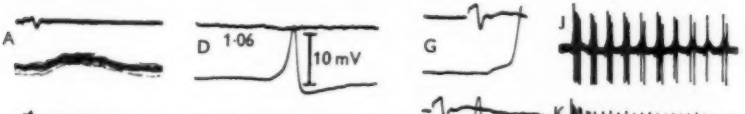

Fig. 12. Intracellular records of a *CN* type interneurone at a depth of 2·0 mm and with a membrane potential of −40 mV. A–C show records evoked by stimuli to the ventrolateral funiculi of the same side and of progressively increasing strength, each record formed by superposition of about 40 faint traces. D–F show responses to a single superficial peroneal volley at the indicated strengths relative to threshold. Upper traces in A–F are records from the L 7 dorsal root entry. G–I show single responses to a descending volley evoked by a stimulus of the same strength as in C, the EPSP being just at threshold for evoking a spike. In G, H there is also a surface record (above) from the spinal cord dorsum, while in I there is an electrically differentiated record of the spike potential (below). Same time scales for all but record I (shown above it); one potential scale for A–C and another for D–I. J–L show responses of another C type cell to repetitive stimulation of the superficial peroneal nerve at frequencies of 22, 67 and 150/sec. In the absence of test by a descending cord volley, it was not known if it was a tract cell (*CT*) or an interneurone (*CN*).

the superficial peroneal nerve. In D the spike clearly arose from an EPSP, much as in G and H. This cell had highly specific synaptic connexions, being activated from no other cutaneous or muscle nerves. Several *CN* cells were activated by high-threshold muscle afferents, however, showing a repetitive response after a long latency.

Type *C* cells in general had much less ability to follow high-frequency stimulation of their monosynaptic path than those of types *A* or *B*. For example, in Fig. 12J–L the type *C* cell responded repetitively at high frequency to a single afferent volley in superficial peroneal nerve (cf. Fig. 10B–D). At a frequency of 31/sec (J) the cell usually responded twice to each afferent volley, and after an initial response at higher fre-

quency it followed 67/sec almost perfectly (K). It failed quickly at 150/sec (L), however, and it also failed to follow 100/sec after the first twelve volleys, showing intermittent following thereafter.

DISCUSSION

In many respects these investigations on the responses of individual interneurones agree with previous investigations in which intracellular recording was used. With the exception of the special cutaneous tract cells (type *CT*) the membrane potential has been much less well sustained than with motoneurones (cf. Haapanen *et al.* 1958; Hunt & Kuno, 1959*a*; Kostyuk, 1959), though it has often been in the range -40 to -60 mV.

There is also general agreement that activated excitatory synapses produce brief depolarizations, the excitatory post-synaptic potentials (EPSPs) closely resembling the EPSPs of motoneurones (Frank & Fuortes, 1956*a*; Eccles *et al.* 1956; Kolmodin & Skoglund, 1958; Haapanen *et al.* 1958; Hunt & Kuno, 1959*a*). Sometimes these EPSPs had a rapid rise and a slower, approximately exponential, decay, as in Figs. 1C; 2A–D; 9O, corresponding to Hunt & Kuno's (1959*a*) description, and also resembling monosynaptic EPSPs of motoneurones. For example, the EPSPs of Fig. 2A–D closely resemble motoneuronal EPSPs even in respect of the half-time of decay, which was about 4 msec. The EPSPs often had a more complex form, however, which is clearly the result of temporal dispersion in the synaptic bombardment (cf. Haapanen *et al.* 1958; Hunt & Kuno, 1959*a*). This was particularly evident when the synaptic bombardment tended to be synchronized into two phases, as in Fig. 1E, F. An extreme temporal dispersion of the synaptic bombardment produced by a single afferent volley is well illustrated in Fig. 3, and can be assumed to be responsible for the prolonged spike discharges of Figs. 6A–G, 10B–D and 11C, E, F. Evidently this synaptic bombardment occurred after transmission through polysynaptic pathways of varying degrees of complexity. The wealth of pathways converging on interneurones is well illustrated by the synaptic noise (cf. Brock, Coombs & Eccles, 1952) produced by continuous random bombardment (cf. Figs. 1A–M, P, Q; Kolmodin & Skoglund, 1958; Haapanen *et al.* 1958).

An analysis of simple EPSPs such as those of Figs. 1C, 2A–D, 9O, P is not possible, because no measurement of the electric time constant of the membrane has been made. Hunt & Kuno (1959*a*) attempted to determine this constant from the strength–duration curves for interneurones, so deriving times with a mean value of only 0·46 msec. With motoneurones, however, this method has given values very much briefer than those derived from the time course of potential changes produced by the application of a rectangular pulse (Frank & Fuortes, 1956*b*; Coombs, Curtis &

Eccles, 1959). Prolonged EPSPs such as those of Fig. 3 would arise by summation of the temporally dispersed unitary EPSPs.

In the present intracellular investigations the spike potential always arose from an EPSP, so it can be presumed that the micro-electrode was inserted into the soma of the neurone and not into the axon, as was sometimes the case with previous investigations (Frank & Fuortes, 1955; Haapanen *et al.* 1958; Hunt & Kuno, 1959*a*). With interneurones of types *A* and *B*, the spike was simple and brief (0·4–0·8 msec), without any trace of the double (IS–SD) composition characteristic of motoneurones (cf. Figs. 1N, O; 2G–J; 9P–T); and this agrees with Haapanen *et al.* (1958) and Hunt & Kuno (1959*a*). On the other hand, types *CT* and *CN* showed a prominent IS–SD configuration (Figs. 10, 11, 12), which clearly indicated a low-threshold area for spike initiation comparable with that of motoneurones (Araki & Otani, 1955; Fatt, 1957; Fuortes *et al.* 1957; Coombs *et al.* 1957*a*; Kostyuk, 1959), and correspondingly the spike complex had a longer duration, about 1 msec. Usually the sizes of spikes were low as a result of cell injury, but overshoot, as in Fig. 10, has been observed by other investigators.

There was a very wide range in the threshold depolarization for initiating a spike discharge. As would be expected, the threshold was high in types *A* and *B*, where there was no specialized low-threshold area. For example in Figs. 1, 2 and 3, it ranged between 8 and 15 mV, while for type *C* cells in Figs. 10–12 the range was 4–8 mV. The range for types *A* and *B* is higher than that (2·5–10·9 mV) reported by Hunt & Kuno (1959*a*). The intensity of excitatory synaptic action appears to be higher with cells of types *A*, *B* and *C* than with motoneurones. Not only is the synaptic depolarization very large as a rule, but it produces high-frequency discharges, as in Figs. 6, 8R, 10, 11; and discharges of the *A* and *B* type cells, can be driven in phase with afferent volleys of very high frequency (Figs. 5, 6, 7, 8). Figure 10P also illustrates the capacity of the EPSP to generate an impulse at only 1·2 msec after the initiation of an antidromic spike.

There is general agreement that the spike declines on to a brief phase of hyperpolarization, such as generally occurs when the membrane potential is low. However, longer after-hyperpolarizations were not found by previous investigators (Haapanen *et al.* 1958; Hunt & Kuno, 1959*a*; Kostyuk, 1959), and were certainly less prominent than with motoneurones (cf. Kolmodin & Skoglund, 1958). *CT* cells by contrast had an after-hyperpolarization as well developed as motoneurones (Fig. 10G, H). With types *A* and *B* there was also evidence of a long after-hyperpolarization (Figs. 2K, 9Q).

A considerable proportion of *A* and *B* cells can follow quite high

frequencies in afferent volleys, up to 700/sec being followed for as many as 30 responses (Figs. 5, 6, 7, 8). The same cell may vary greatly in its responses to repetitive volleys from different afferents, which is satisfactorily accounted for by the relative excitatory potencies of the different volleys (cf. Figs. 7, 8). No distinction can be made between the frequency responses of type *A* and *B* cells. Such high-frequency following of interneurones has not been reported by other investigators (cf. Frank & Fuortes, 1956*a*; Haapanen *et al.* 1958). Possibly their investigations were largely restricted to the interneurones activated only through polysynaptic paths, which we have not classified. Many of these give high-frequency responses (up to 1000/sec) to single volleys, but follow repetitive afferent volleys very poorly (e.g. Fig. 12J–L). In some cases the mixed afferent volleys had an inhibitory action which cumulatively depressed the responses to the later volleys (cf. Hunt & Kuno, 1959*b*). At least four factors are probably concerned in the high frequency with which type *A* and *B* cells can follow repetitive synaptic bombardment: the high level of monosynaptic excitatory action; the brevity of the spike and consequently of the refractory period (cf. Fig. 9J); the relatively low level of the afterhyperpolarization; and, finally, the high-frequency potentiation of EPSPs (cf. Curtis & Eccles, 1960) may be as well developed with interneurones as it is with many cells of origin of the ventral spinocerebellar tract (Eccles, Hubbard & Oscarsson, unpublished observations).

The primary afferent fibres from which a cell draws its monosynaptic activation have been a relatively unambiguous guide to the definition of cell type, so that there are numerous specimens belonging uniquely to one or other of the three main types *A*, *B* or *C*. There have been four interneurones, however, which could not be classified as *A* or *B*. For the present it may be assumed that this ambiguity was due to inadequate investigation and to unfavourable threshold discrimination between the Ia and Ib afferent fibres of the muscle nerves. It can be expected that the much more selective conditions provided by adequate stimulation will allow the complete separation into types *A* and *B*, just as with the cells of origin of the dorsal spinocerebellar tract (Lundberg & Winsbury, 1960*a*, *b*). If further investigation confirms this prediction, there would be further support for the general proposition that the primary afferent fibres belonging to Groups Ia and Ib do not establish synaptic connexions on the same nerve cell. There are already several well established instances of this principle of exclusion. Ia fibres alone make direct synaptic connexions with motoneurones (Bradley & Eccles, 1953; Eccles *et al.* 1957*a*) and with the interneurones (a subgroup of type *A*) concerned in direct inhibitory action (Eccles *et al.* 1956; Eccles *et al.* 1957*a*). Furthermore, the cells of origin of the dorsal spinocerebellar tract activated by Ia and Ib fibres now

appear to be distinct (Lundberg & Winsbury, 1960*b*). Finally, the Ib afferent fibres are alone concerned in activation of the cells of origin of the ventral spinocerebellar tract (Oscarsson, 1956, 1957). It would seem likely that in the development of all these specific central connexions of Ia and Ib afferent fibres there is some chemical specificity in the respective growing fibre tips or growth cones. This postulate would be of particular significance in accounting for the specific afferent connexions made on the *A* and *B* cells interspersed in the intermediate nucleus.

The destination and functional action of the axons of the nerve cells give a basis for further classification. This criterion is well established for such spinal nerve cells as motoneurones, and the cells of origin of the dorsal and ventral spinocerebellar tracts. It has been applied above in subdividing type *C* cells into *CT* and *CN* subtypes, but it also can be applied to cells of types *A* and *B*.

It is now necessary to postulate that Ia afferent fibres exert not only a monosynaptic, but also a disynaptic or polysynaptic excitatory action on some interneurones (Fig. 1G, H) and motoneurones (Fig. 4A–F). Both general considerations and specific investigations have led to the hypothesis that nerve cells are either excitatory or inhibitory in action (Eccles, 1957). Hence it would be predicted that type *A* cells could be subdivided into two sub-types according as they are excitatory or inhibitory in function, i.e. sub-types *AE* and *AI*, respectively. Possibly the *AI* cells are those with the very restricted receptive field, in conformity with their special function as interneurones of the very specific direct inhibitory path (cf. Fig. 5), whereas type *AE* might include the cells with wider receptive fields (Figs. 1, 2, 6). Further investigation is desirable in order to see if the cells of type *A* can be subdivided in this way in relation to their receptive fields.

Since the disynaptic inhibitory action of Group Ia impulses is much more in evidence than di- or polysynaptic excitatory actions, it would be expected that type *AI* would be more numerous than type *AE*. Impulses passing down the descending tracts of the spinal cord profoundly depress the interneuronal pathways from primary afferent fibres to motoneurones, with the sole exception of the 'direct' inhibitory path (Eccles & Lundberg, 1959*a*; Holmqvist & Lundberg, 1959). It would therefore be expected that such descending inhibitory action would distinguish other interneurones from *AI* cells.

Similar considerations apply to type *B* cells, for Ib afferent impulses have a powerful disynaptic inhibitory action on motoneurones and also have powerful disynaptic or polysynaptic excitatory actions (Laporte & Lloyd, 1952; Eccles *et al.* 1957*c*). Hence it may be postulated that there are many cells in each of the subtypes *BI* and *BE*.

The type *CT* cells have the properties which would be expected for the cells of origin of the cutaneous component of Fleschig's fasciculus (Morin, 1955; Laporte *et al.* 1956*a*, *b*; Catalano & Lamarche, 1957) which makes synaptic connexion with the lateral cervical nucleus (Rexed & Ström, 1952). Recording from the fibres of this tract (Laporte *et al.* 1956*a*, *b*) shows that the cells are activated monosynaptically by the lowest threshold cutaneous fibres, the synaptic delay being less than 1 msec, while larger cutaneous volleys cause the repetitive discharge of several impulses with a frequency as high as 1000/sec, exactly as in Figs. 10 and 11. The fast conduction velocity of the axons of this tract, 100 m/sec (Laporte *et al.* 1956*a*; Oscarsson, 1958), indicates that it arises from large cells, which agrees with the tolerance of intracellular electrodes shown by *CT* cells. This cutaneous tract would include the dorsal spino-olivary tract described by Grundfest & Carter (1954); but there are minor discrepancies, the conduction velocity (maximum at 75 m/sec) being considerably lower (di Biagio & Grundfest, 1955). Possibly some systematic error was responsible for this discrepancy, because this conduction velocity was linked with a localization of the tract cells to the L5 segmental level, whereas the *CT* cells were actually at the L7 level in our experiments.

The *CT* cells have been located at depths of 1·6–2·0 mm from the cord dorsum, except for one at 2·5 mm, being thus more superficial than the intermediate nucleus. There was a corresponding location (maximum at 1·7 mm depth) for the synaptic field potential evoked by low-threshold cutaneous volleys (Coombs *et al.* 1956). Presumably the *CT* cells are the large neurones reported by many investigators in the deeper part of the dorsal horn, the 4th and 5th layers of Rexed (1952, 1954). Lenhossék (1893) and Cajal (1909) both give illustrations showing the axons of some of these neurones passing into the lateral columns. The *CN* cells have been scattered more widely, their depths ranging from 1·2 to 2·7 mm in our few samples. The depth of the *A* and *B* type cells would place them in the 6th layer of Rexed (1952). Certainly they are far too widely dispersed for location in the very slender nucleus intermedio-medialis. On the other hand Cajal (1909, Fig. 115) has depicted a very dense plexus of collaterals of primary afferent fibres in the precise region where *A* and *B* type cells are found. Clearly further work is needed to correlate cytoarchitectonics with synaptic connexions and electrophysiological investigations.

In addition to the 90 neurones of the *A*, *B* and *C* types, about 30 other interneurones were investigated, but could not be classified. Four were monosynaptically activated by Group I fibres from muscle nerves, but the data were inadequate for separating them into *A* and *B* types. The remaining unclassified neurones were not monosynaptically activated from any of the muscle or cutaneous nerves prepared for stimulation. No doubt

many would have been identified as belonging to one or other type if they had been tested by all muscle and cutaneous nerves of the hind limb. It should also be pointed out that we have not defined any neurones by the criterion of monosynaptic activation by Group II afferent fibres from muscle. Nevertheless, it would seem likely that some interneurones have no monosynaptic activation from primary afferent fibres. Another type of interneurone, recently found in the dorsal horn (Eccles & Krnjević, 1959), is characterized by very large (up to 40 mV) and prolonged (200 msec) EPSPs without any detectable spike potential.

SUMMARY

1. Micro-electrode techniques have been employed in studying types of neurones which are located in a restricted zone in and around the intermediate nucleus of the spinal cord from lower L6 to upper S1 levels.

2. The receptive fields from which these neurones exclusively draw their monosynaptic innervation have been the basis of their subdivision into three main types, about 30 of each type having been investigated: Ia afferent volleys and type *A*; Ib afferent volleys and type *B*; cutaneous afferent volleys and type *C*. Many neurones could not be so classsified; some because the data were inadequate, but many because they had no monosynaptic activation.

3. The axons of some type *C* cells ascend the cord in the ventrolateral funiculus of the same side, i.e. they are cutaneous-tract cells. The axons of type *A* and *B* cells and the remaining type *C* cells do not ascend the cord. The cells would be interneurones taking part in spinal reflexes or mediating actions to ascending pathways.

4. Intracellular recording showed that synaptic excitatory action evoked discharge by a depolarizing action (the EPSP) which was often large.

5. Besides their monosynaptic excitation many cells of all types have polysynaptic innervation from muscle and cutaneous nerves, which may give high-frequency discharge up to 1000/sec, particularly from *C* type cells.

6. The spike potentials of type *C* cells have a double composition resembling the IS–SD spike of motoneurones. With cells of types *A* and *B* the spike was simple, so it is presumed that they have no low-threshold area for initiating spikes.

7. Type *C* cells have a large and prolonged after-hyperpolarization, and so possibly do *A* and *B* cells; nevertheless, on account of the powerful monosynaptic action, *A* and *B* type cells can follow high frequencies of afferent volleys, even up to 700/sec.

8. There is a brief discussion of problems which arise in attempting to account for the development of specific central connexions by primary afferent fibres.

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ABOMASAL SECRETION IN THE SHEEP

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Anatomically and histologically the true gastric secretory stomach of the ruminant, the abomasum, is strictly comparable to the simple stomach of other mammals. The composition of the gastric juice is similar to that found in other animals and it is reasonable to assume that the basic mechanisms governing secretion are also essentially similar. The cranial part of the ruminant digestive tract is, however, considerably modified for the storage and fermentation of plant material and it is of interest to ascertain to what extent the function of the abomasum is modified by the three compartments of the forestomach which lie between it and the oesophagus. Previous investigations (Hill, 1955) have shown that the continuous flow of ingesta from the reticulo-rumen to the abomasum is responsible for its continuous secretory activity, and that the abomasal glands are not able to secrete spontaneously. The experiments to be described were carried out to obtain further information about the factors responsible for initiating and maintaining abomasal secretion.

METHODS

Surgical preparations

One- to two-year-old Clun Forest sheep were taken from pasture and placed in individual pens in the animal house, where they were allowed a week or two to become acclimatized before operation. Mineral supplements and a daily ration of 1000 g of chopped hay were provided for all animals.

Rumen fistulae were prepared in all animals and were fitted with polyvinyl cannulae (Jarrett, 1948).

Abomasal fistulae. Perspex cannulae, 16 mm diameter and fitted with screw caps, were inserted into the pyloric region of the abomasum, and brought out through the abdominal wall.

Crocker–Markowitz fistula. The duodenum was transected immediately caudal to the pyloric sphincter and a stainless-steel cannula (external diam. $\frac{7}{16}$ in. (11 mm), internal diam. $\frac{6}{16}$ in. (9·5 mm)) inserted into each end. The stems of the cannulae were brought out through the body wall and continuity of the digestive tract re-established by connecting the two cannulae with plastic tubing (Dougherty, 1955).

Vagally innervated abomasal pouches were prepared in nine sheep by a modified Hollander technique (Gregory, Hallenbeck & Code, 1942). Perspex or stainless-steel cannulae with

stems of 16 mm diameter were placed in the pouches and brought out through stab wounds in the abdominal wall. The difficulties associated with the preparation of gastric pouches in ruminants have been discussed elsewhere (Hill & Gregory, 1951; Masson & Phillipson, 1952) but attention should again be directed to the importance of securing adequate drainage of the pouches, since the many large folds of mucosa frequently block the pouch end of the cannula and interfere with the collection of gastric juice. This difficulty was overcome in the present work by placing in the pouch a soft polyvinyl drainage tube made in the form of a perforated hollow sphere approximately 4 cm in diameter and with a stem 8 cm long. The drain was inserted into the cannula and pushed up into the pouch in order to hold the mucosal folds apart and so allow the gastric juice to drain freely. The experimental observations described here were made several months, and in some cases a year or longer, after the pouches had been made.

Although a few experiments were carried out in the animal house the sheep were usually taken to the laboratory and placed in stocks. After a time they became accustomed to this procedure and would stand quietly in the stocks for many hours. Half-hourly samples of gastric juice were collected in a graduated centrifuge tube attached to a small plastic funnel; this was held under the pouch cannula by a cord secured around the sheep's body.

Oesophagostomy. Two sheep were operated upon by the two-stage method described by Friedman (1951). In one sheep (No. 30) oesophagostomy with a 5-day interval between Stage I and Stage II was performed 3 months after an abomasal pouch had been prepared. In the other one (No. G 103) Stage I was performed before making the pouch and Stage II 3 months afterwards; both animals recovered rapidly from the second stage of the oesophagostomy and ate well for several days, the food boluses being collected as they dropped from the oesophagostomy and returned to the animal through the rumen fistula. When this operative technique is used in the dog, the sides of the oesophagostomy can be closely approximated by placing a bandage around the neck, and the animal can then eat normally. In the sheep, although the edges of the incision were brought together in a similar manner, food boluses were always ejected during eating. This may have been because the boluses, which were 8–10 cm long and 25–35 mm in diameter, were too bulky to pass through the slightly narrower oesophagus at the operation site. Saliva was lost continuously from the oesophagostomy and although this was collected and returned to the rumen the loss may have been a factor which contributed to the decline in general condition which occurred in both animals 4–5 days after operation. Both animals attempted to ruminate but this merely resulted in the loss of regurgitated rumen contents through the oesophagostomy. Sham-feeding experiments were therefore done on these animals during the first few days after the oesophageal stomata had been established.

Venous catheterization. Small-bore (external diam. 1·5 mm, internal diam. 1·0 mm) polythene tubing was introduced through a wide-bore needle into the jugular vein and held in position with sticking plaster for the duration of each experiment. Heparinized saline (10 u./ml. Liquemin; Roche Products, Ltd.) prevented clot formation in the catheter.

Analytical

pH was determined with a glass electrode (Pye Universal pH Meter, Cambridge). Free and total acid of the gastric juice was determined by electrometric titration with 0·1 N-NaOH to pH 3·5 and 9·6, respectively. Peptic activity of the gastric juice was estimated by Hunt's (1948) method. Blood sugar was estimated by the Shaffer–Somogyi method (Shaffer & Somogyi, 1933). Volatile fatty acids were determined by steam distillation, with precautions to exclude CO_2, and titration of the distillate with carbonate-free 0·02 N-NaOH and phenolphthalein as indicator (Annison, 1954).

RESULTS

The effect of a meal on secretion from abomasal pouches

Secretion of gastric juice was first observed under feeding conditions which simulated, as far as possible, those at pasture. The animals remained in their pens in the animal house and consecutive hourly collections of juice were made. Food was provided *ad lib.* and was eaten by the animal intermittently during the day. Under these conditions, although there was continuous secretion of acid gastric juice, there were minor fluctuations in the amount and acidity of the juice secreted each hour and an over-all trend towards higher secretory rates between 5.0 a.m. and 2.0 p.m. Over a 24-hr collection period the average hourly output of free acid was usually about 1·70 m-equiv, and of total acid about 1·90 m-equiv. Comparatively large volumes of gastric juice were secreted by the pouches; sheep H 149 for example, produced an average of 526 ml./day with a total acidity of 88·5 m-equiv/l. during a collection period of 12 days. If, as seems likely from inspection of its area, the pouch mucosa formed about 12 % of the total secretory mucosa of the abomasum, the total daily production of gastric juice would be of the order of 4·0–4·5 l.

When animals were deprived of food for 24 hr, the volume and acidity of the abomasal secretion were reduced although there were still minor fluctuations in the rate of secretion. Feeding was then followed by a marked increase in secretion and this was found to be even more pronounced in sheep which had been in the animal house for long periods; possibly because these animals ate a large proportion of their daily ration in a very short space of time. Figure 1 illustrates the secretory response obtained when 400 g of dried grass cubes were eaten during a half-hour period. The first 30-min sample showed an increase in the volume and acidity of the juice and this became maximal 1–1½ hr after the start of feeding, gradually subsiding over the next 3 hr. When chopped hay or green stuff was eaten a similar secretory response was obtained but it tended to be short-lived. Collection of juice at 10 min intervals during the feeding response showed that the onset of secretion occurred within 10 min of the start of feeding and became maximal in about 60 min. Occasionally in the feeding experiments secretion was depressed during the first 30 min after feeding. This was particularly so if the secretory rate before feeding was at a relatively high level.

Although similar increases in volume and acidity of the juice were obtained in response to feeding from all the sheep with pouches, there were individual differences in pepsin secretion. Thus in Sheep 24 (Fig. 1) the pepsin concentration remained practically unchanged during the feeding response, although the output was naturally elevated when the secretory

rate was high, maximum output being obtained during the early stages of the secretory response. Sheep H.160 and 37, on the other hand, showed an appreciable increase in the concentration of pepsin in the gastric juice and consequently a greater output of pepsin. The maximum increase in pepsin concentration occurred 1–1½ hr after the start of feeding and coincided with peak volume and acid output. In two further sheep, K94 and 30, the pepsin concentration and output although increased, were less than those observed in Sheep H.160 and 37. Atropine (10 mg subcutaneously) completely inhibited the feeding response.

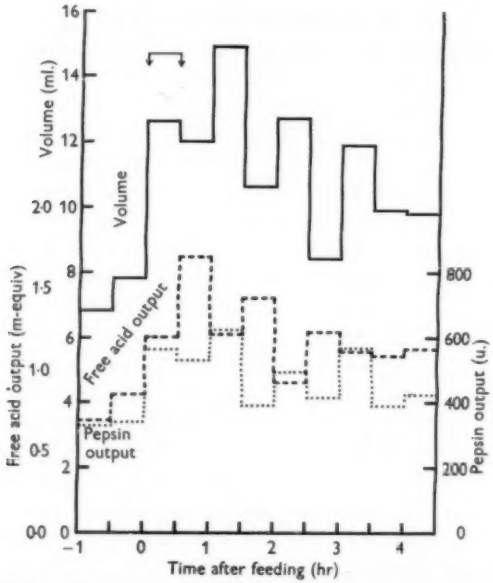

Fig. 1. The effect on abomasal secretion of eating a meal (400 g dried grass cubes, between arrows). Sheep 24. Modified Hollander pouch.

Another feature observed in animals which had become accustomed to the feeding regime was what appeared to be a conditioned secretory response to feeding. Thus, when an animal had been fasting for 24 hr and the secretory activity of the abomasum had fallen to a low level, there was sometimes a transient increase in secretion at the usual feeding time. This response was not observed when the reticulo-rumen had previously been emptied of ingesta. Feeding other sheep in the presence of the experimental subject occasionally resulted in a slight increase in abomasal secretion as did the sight and smell of food.

The effect of vagal denervation on abomasal secretion was studied in four sheep with Heidenhain pouches. In all these animals the amount of juice secreted in response to a meal was considerably less than from innervated pouches of comparable size, and it was frequently of very low acidity. The time relations of the secretory response to a meal were, however, comparable to those of an innervated pouch.

The flow of ingesta during feeding

The rate of reticulo-rumenal contractions increases when a meal is eaten and it seemed probable, therefore, that there would be a concomitant increase in the flow of ingesta into the abomasum, which might be responsible for the secretory response observed after feeding. This point was investigated in Sheep G 93, which had a modified Crocker–Markowitz fistula. During experiments the connecting tubing between the two cannulae was removed and the ingesta which issued from the cranial cannula were collected and measured. They were then returned to the duodenum through the second cannula. When the animal was deprived of food the flow of ingesta from the cannula adjacent to the pylorus was greatly reduced, but eating a meal then provoked an increase in flow which was maximal during the first hour after the start of feeding. This was followed over the next 3–4 hr by a gradual reduction to the pre-feeding level (Fig. 2).

The passage of relatively large volumes of ingesta into the abomasum demonstrated in this way would presumably bring about some degree of distension of this organ and since distension of the pyloric antrum is known to stimulate gastric secretion in other animals further experiments were carried out in which the effect of pyloric distension on abomasal secretion was investigated. Animals prepared with abomasal pouches and pyloric fistulae were used and the pyloric region was distended by the inflation (40 ml. air) of a balloon passed into the abomasum through the fistula. The results were variable but in three experiments out of six secretory responses were provoked by moderate distension of the pylorus (Fig. 3). Ash (1959) has recently reported that distension of the main body of the abomasum with a balloon containing 250–500 ml. 0·9 % NaCl solution also stimulates the secretion of gastric juice.

The effect of sham-feeding on abomasal secretion

Several feeding experiments were done on Sheep 30 and Sheep G 103 before oesophagostomy and in all of them typical secretory responses were obtained. After oesophagostomies had been established four 'sham-feeding' experiments were performed whilst the animals were still in good condition. In none of them was a secretory response obtained. It is well

known, however, that in the dog the 'sham-feeding' response is capricious and easily inhibited by pain or apprehension and therefore the negative evidence obtained in the sheep did not necessarily constitute very good proof of the absence of a cephalic phase of secretion. Because of the peculiar nature of the ruminant digestive tract there is, however, no real need to perform oesophagostomy in order to observe 'sham-feeding', since a form of this may be simulated by emptying the reticulo-rumen. Because of the large capacity of the reticulo-rumen, food, when eaten, then merely

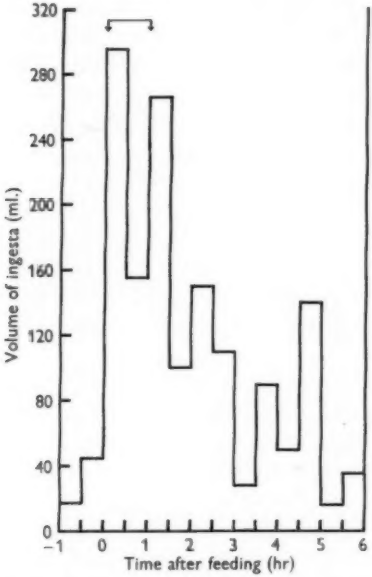

Fig. 2. The effect of eating a meal (500 g chopped hay, between arrows) on the flow of ingesta from the abomasum. Sheep G.93. Crocker–Markowitz fistula.

passes to the rumen and remains there for several hours without apparent change, providing the forestomachs have been completely emptied and washed out. Further experiments were therefore carried out in which such a preparation was used. After the reticulo-rumen had been emptied and washed free of ingesta the secretion from the abomasal pouches was much reduced in volume and contained no free acid. Food was then given to the animals and by observation through the rumen fistula it was possible to see the swallowed boluses entering the rumen where they gradually accumulated. The pouch secretion continued to be scanty and did not contain any free acid (Fig. 4). This was in marked contrast to the large secretory

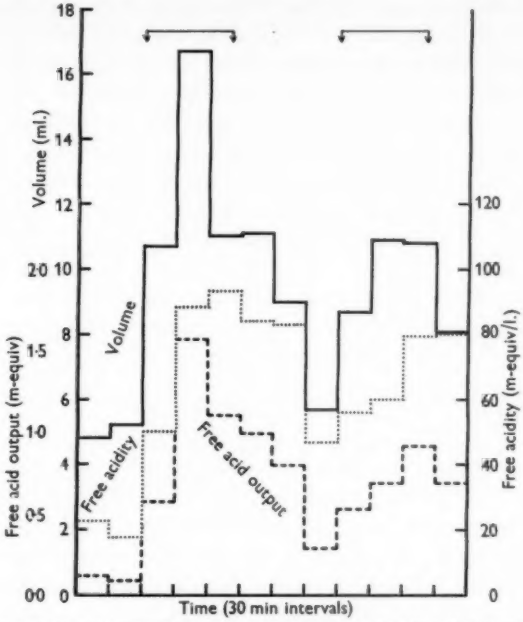

Fig. 3. The effect on abomasal secretion of distension of the pyloric region of the abomasum; between arrows, pylorus distended with balloon (40 ml. air). Sheep 42. Modified Hollander pouch.

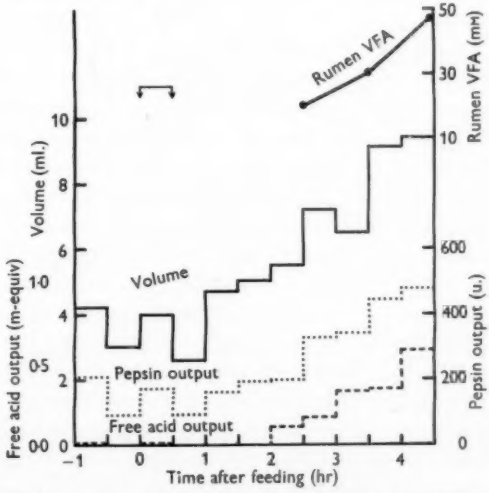

Fig. 4. The effect on abomasal secretion of eating a meal, 150 g chopped hay (between arrows); reticulo-rumen empty of ingesta. Sheep H.160. Modified Hollander pouch.

responses to feeding obtained from the same animals when the forestomachs contained ingesta. No effect was observed on pepsin secretion in any of the animals, even though two of them, H.160 and 37, had shown increases in both concentration and output of pepsin after normal feeding. In several of these experiments there was a slight to moderate increase in secretion some 2–2½ hr after feeding. Since 'sham-feeding' and feeding with the reticulo-rumen empty failed to provoke a secretory response from the abomasum it appeared that the large increase in secretion observed after normal feeding was caused by an accelerated flow of ingesta into the abomasum. There was, however, marked stimulation of pepsin secretion in some of the feeding experiments and as pepsin secretion is generally considered to be under nervous control it seemed desirable to investigate further the possibility of vagal participation in the secretory response.

The effect of vagal stimulation on abomasal secretion

Vagal stimulation brought about by insulin hypoglycaemia has been employed extensively in studies in gastric secretion (see Bachrach, 1953) and although previous investigations (Hitchcock, Karvonen & Phillipson, 1948; Hill, 1952*b*) had indicated that insulin hypoglycaemia was inhibitory to abomasal secretion it was deemed worth while to reinvestigate this point.

Sheep H 160 and 37 were deprived of food for 24 hr and a fine polythene catheter was inserted into the jugular vein immediately before the experiment. This made it possible to give injections and to remove blood samples with minimal disturbance to the animal. After a control period insulin (Burroughs Wellcome, 40 u./ml.) was injected intravenously (0·5 u./kg). For the next 30 min there was either no change or a slight depression in secretion, but thereafter the volume and acidity increased and reached maximum values 1–2 hr later. This secretory response coincided with the fall in blood sugar and was maximal at the time of most pronounced hypoglycaemia. The pepsin concentration in the juice was slightly depressed immediately following the insulin injection and then gradually increased until it was approximately four times greater than the pre-injection level. Pepsin output was markedly elevated during the maximal volume response (Fig. 5). Although these results suggested that gastric secretory fibres were present in the vagi, another possibility was suggested by the results of previous investigations, which have shown that insulin hypoglycaemia also produces supermotility of the rumen and reticulum (Le Bars, Nitescu & Simmonet, 1953; Hill, 1954). It might therefore be that the insulin-stimulated secretion was caused by an augmented flow of ingesta to the abomasum. This point was examined in experiments in which the passage of ingesta to the abomasum was prevented by emptying the reticulo-rumen before the administration of insulin.

As in the experiments described previously the pouch secretion, after the forestomachs had been emptied, was reduced in volume and contained little or no free acid. After the intravenous injection of insulin (0·5 u./kg) secretion of juice was depressed for 30–60 min, and was then only slightly stimulated. However, the pepsin concentration of this juice was again increased to about four times the pre-injection value, although the output was a great deal less than that obtained when the reticulo-rumen contained ingesta (Fig. 6).

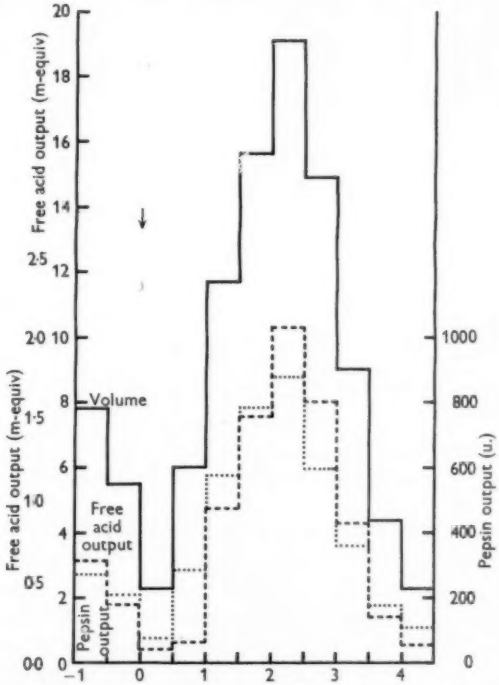

Fig. 5. The effect on abomasal secretion of insulin 0·5 u./kg I.V. at arrow. Reticulo-rumen containing ingesta. Sheep H.160. Modified Hollander pouch.

Carbamyl-choline chloride (Carbachol; British Drug Houses) given intravenously in a dose of 0·5 mg to two sheep with abomasal pouches caused a pronounced increase in the volume, acidity and pepsin concentration of the juice and in the output of acid and pepsin. The same dose of carbachol given to these sheep after the reticulo-rumen had been emptied produced comparable stimulation of pepsin secretion but a smaller acid and volume response.

Volatile fatty acids and abomasal secretion

On completion of those experiments in which the reticulo-rumen had been emptied it was the usual procedure to replace the ingesta through the rumen fistula. Whilst doing this it was noticed that there was frequently a sudden enhancement of abomasal activity. Although the obvious explanation was that this was brought about by the resumed flow of ingesta to the abomasum, it seemed possible that fermentation products present in the ingesta might have stimulated secretion after they had been absorbed from the rumen.

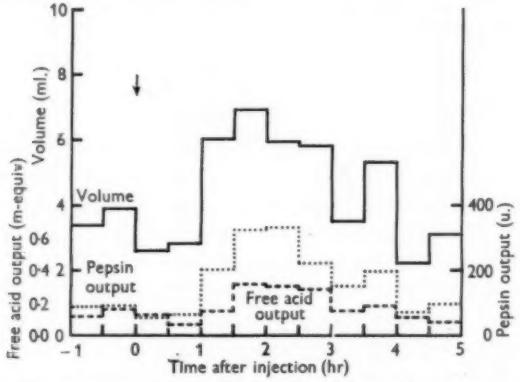

Fig. 6. The effect on abomasal secretion of insulin 0·5 u./kg I.V. at arrow. Reticulo-rumen empty of ingesta. Sheep H. 160. Modified Hollander pouch.

Confirmation of this idea was obtained from certain of the experiments in which sheep were given a meal after the reticulo-rumen had been emptied. As described previously there was, in some of these experiments, slight stimulation of secretion 2–2½ hr after feeding. Because of this time lag the secretion then occurring was unlikely to have been associated with the act of eating, and since the food boluses, when swallowed, entered the ventral sac of the rumen and remained there it was also unlikely that any material could have passed on to the abomasum. It was noted, however, that the food boluses lying in the ventral sac gradually lost their discrete nature and became semi-solid in consistency. This was probably partly because of their admixture with saliva but it may have been because some degree of bacterial fermentation had occurred. It is difficult to remove through a relatively small rumen fistula all traces of ingesta from the rumen and the changes observed might well have been brought about by bacteria which had remained in the rumen. As an indication, therefore, of whether or not fermentation had actually occurred in the rumen the

concentration of volatile fatty acid (VFA) was determined in the ingested food material. In the 2 hr after feeding the food boluses remained relatively solid and it was only after this time that it was possible to obtain adequate fluid samples for VFA estimation. Their concentration was then found to increase progressively. It was during this period that the increase in secretion from the abomasal pouch occurred (Fig. 4).

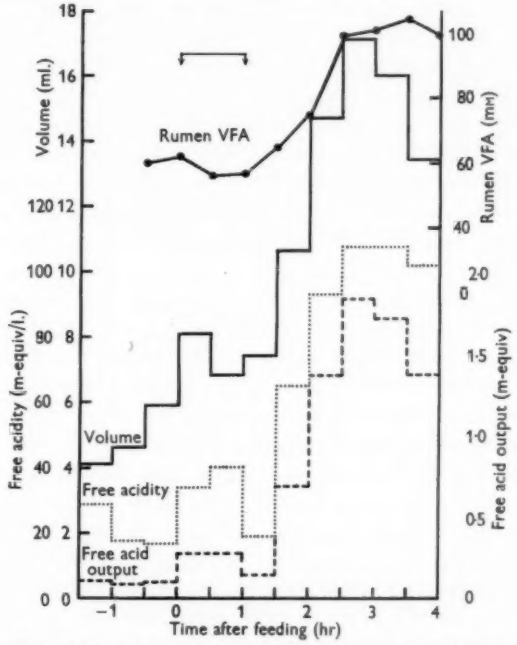

Fig. 7. The effect of eating a meal (between arrows) on abomasal secretion and on the concentration of volatile fatty acid (VFA) in the rumen: the meal was 380 g chopped hay + 200 g oats. Sheep K 94. Modified Hollander pouch.

Because of this apparent correlation between the increase in concentration of rumen VFA and the increase in abomasal secretory activity in sheep with the rumen empty the rumen VFA and abomasal secretory patterns were compared in normal feeding experiments (Fig. 7). In most of these experiments in which hay and oats were fed it was usual for the rumen VFAs to increase rapidly to between 100 and 120 m-mole/l. during the 2–3 hr after feeding and then to decline slowly over the subsequent few hours. For the first 1–2 hr after feeding there was always good correlation between the rapid increase in VFAs and the increase in secretion, but after this time there was only rarely any correlation between the two. It was in fact usual for the secretory response to subside about this time

while the VFA remained high or declined only slightly. Neither does there appear to be any relation between the actual concentration of VFA in the reticulo-rumen and the rate of secretion from the abomasum, since considerable secretion occurred after kale had been eaten when the rumen VFAs remained relatively low.

In an attempt to separate the secretory effects of an increased flow of ingesta to the abomasum caused by eating from possible stimulatory effects of VFAs, food was placed directly in the rumen through the rumen fistula. Five such experiments were carried out and in each of them there was a small to moderate secretory response. VFA production usually increased more slowly than after normal feeding and maximum concentration was not attained until some 3–4 hr after food had been placed in the rumen. Abomasal secretion followed a similar time course.

These experiments indicated that fermentation products, possibly fatty acids, might act as stimulants to gastric secretion, and consequently the effect of adding individual fatty acids to the rumen contents was investigated. Figure 8 shows an experiment in which three separate doses of 250 m-mole of acetic acid neutralized to pH 5·5 with NaOH were added to the rumen contents at hourly intervals. The animal was deprived of food for 24 hr before the experiment and during the control collection period the secretion was much reduced in volume and acidity whilst the VFA concentration in the rumen remained steady at a relatively low level. During the 30 min following the addition of the first dose of acetic acid there was considerable stimulation of secretion and this was even greater after the addition of the two remaining doses. The level of total VFA in the rumen increased rapidly to a maximum of 105 mM, which was well within the normal range. One hour after the addition of the last dose of acetic acid there was marked reduction in the secretion even though the VFA concentration in the rumen had not declined. Pepsin concentration and output were increased to approximately twice the control level. Acetic acid adjusted to pH 6·0, 6·2 and 6·5 with NaOH gave similar results, as did the addition of acetic acid to the empty rumen.

Experiments were also done in which similar amounts of propionic acid and butyric acid were added to the rumen. Both of them were found to stimulate abomasal secretion although to a lesser extent than acetic acid. The results with butyric acid were particularly variable, since either good or poor responses were obtained with the same dose of acid.

As it is generally held (Barcroft, McAnally & Phillipson, 1944) that the VFA produced during fermentation is absorbed from the forestomachs and that only small amounts reach the abomasum, it seemed possible that the secretagogue effects of the fatty acids were mediated after they had been absorbed and were not caused by direct stimulation of the gastric

glands after they had passed to the abomasum. Investigations on the VFA content of the abomasal ingesta after the addition of 250 m-mole of acetic acid to the rumen gave support to this view, since this did not result in any significant change in the amount of fatty acid in the abomasum. When, however, larger amounts of acetic acid were added to the rumen there was a marked rise in the VFA concentration in the abomasal ingesta.

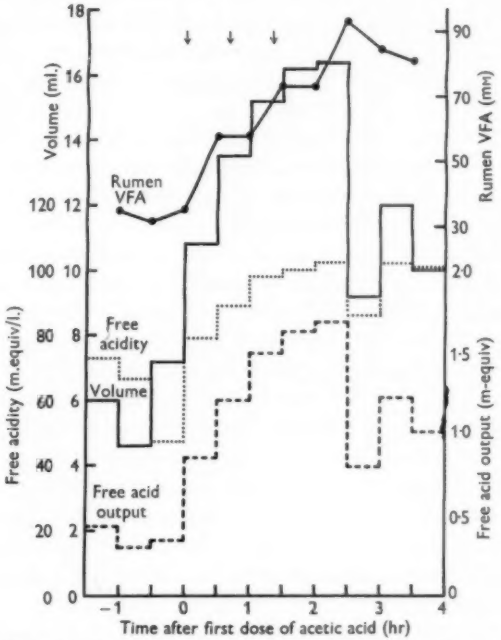

Fig. 8. The effect of adding acetic acid to the rumen contents on abomasal secretion: each arrow marks addition of 250 m-mole acetic acid, pH 5·5. Sheep H. 160. Modified Hollander pouch.

DISCUSSION

Because of the relatively poor food value of plant material ruminants at pasture spend a considerable proportion of each 24 hr eating, and there is therefore frequent addition of fresh food material to the reticulo-rumen where it is subjected to microbial fermentation and whence there is a more or less uninterrupted flow of ingesta through the omasum into the abomasum. It was therefore not surprising to find continuous secretion of highly acid juice in the experiments where natural grazing conditions were simulated by frequent feeding. Under these conditions there was no obvious correlation between feeding and the secretory activity of the abomasum,

findings which are in agreement with those of Masson & Phillipson (1952). The estimated total daily output of gastric juice under these conditions was also in reasonably close agreement with the figures of these authors. Previous investigations (Grosser, 1905; Belgowski, 1912; Hill, 1952*a*, 1959*a*; Kuimov, 1952), also confirmed here, have shown, however, that when food is withheld the level of secretion is reduced and that feeding is then followed by a marked secretory response. Kuimov also observed differences in the volume of juice secreted after different foodstuffs were fed, and this finding has received some confirmation in the present investigations, where the secretory response after hay was less than after concentrate had been eaten. Variations in the gastric secretory response to different foodstuffs have been recognized in monogastric animals for many years, but they are of particular interest in ruminants where ingested food material does not pass directly to the secretory stomach and where the composition of the material which passes to the abomasum is probably of relatively constant composition. In view of the secretogogue effects of fatty acids it may be that differences in the amounts and relative proportions of these substances when concentrates are eaten are responsible for their more prolonged stimulation of abomasal secretion. Long-term experiments with animals on specific diets are needed before the influence of particular foods on abomasal secretion can be fully assessed.

The cephalic phase of gastric secretion in ruminants is generally considered to be of little significance. Thus Popov (1932), who appears to have been the first to suggest that the reflex phase of secretion is not so marked in ruminants, found little difference in the secretory behaviour of vagally innervated and vagally denervated gastric pouches in sheep. Espe & Cannon (1937), who performed oesophagostomies in calves with Pavlov pouches and carried out 'sham-feeding' experiments with milk, also concluded that the psychic or cephalic phase was absent or of little importance.

The 'sham-feeding' experiments reported here also indicate that a cephalic phase of secretion is absent in the sheep and this was confirmed by the fact that the secretory response obtained when normal animals were fed was absent when the forestomachs were devoid of ingesta. These results show that the presence of ingesta in the reticulo-rumen is necessary for the development of a secretory response. Because of the considerable increase in reticulo-rumenal movements during eating and the concomitant increase in the flow of ingesta, it seems likely that this accelerated flow stimulates the pyloric phase of secretion and is mainly responsible for the secretory response. Some supporting evidence for this is provided by the increase in secretion observed after pyloric distension. It is recognized that there are species differences in the relative importance of the different

phases of gastric secretion (James, 1957) and it is perhaps not unreasonable to assume, because of the continuous nature of digestion in the sheep, that there is no necessity for a rapid outpouring of gastric juice of high enzyme content such as is produced during the cephalic phase of secretion in the dog.

The apparent conditioned response observed in some animals at the normal feeding time may be explained by the finding of Quin & Van der Wath (1938) and Dedashev (1959) that the movements of the reticulo-rumen can become conditioned to feeding and that there is supermotility of both chambers at the expectation of food. As the response was not observed when the forestomachs were empty of food it could not have been the result of a conditioned reflex affecting the gastric glands directly. The occasional responses obtained when animals witnessed the feeding of others probably have the same explanation.

The demonstration of a secretory response to insulin hypoglycaemia was at variance with previous results obtained in the lamb (Hitchcock *et al.* 1948) and in the goat (Hill, 1952*b*), where inhibition of secretion was noted. The reason for this discrepancy is not known but the over-secretion which occurred in the present experiments when the reticulo-rumen contained food material indicated that the vagi exert considerable control over the gastric glands. That this control was, however, mainly indirect, was shown by the small response obtained when insulin was given to animals in which the forestomachs were empty of ingesta. These results again indicate that an increased flow of ingesta into the abomasum, provoked by reticular-rumenal supermotility, is the most important factor in the stimulation of abomasal secretion.

The persistence of a small secretory response to insulin hypoglycaemia after the forestomachs had been emptied did, however, show that some secretory fibres were present in the vagi. The lack of response to sham-feeding with the rumen empty therefore suggests that, if there is a vagal component in the normal secretory response to feeding, then it must be mediated through afferents so situated that they are not stimulated by sham-feeding. Such would be the case if receptors were located elsewhere than the buccal cavity and in this respect it may be pertinent to note that sensory nerve endings have, in fact, been demonstrated in the reticulo-rumen epithelium (Hill, 1959*b*). Whether these are concerned in the reflex control of gastric secretion remains to be seen but it is of interest that vagal afferents concerned with salivary secretion have already been described in the reticulo-rumen (Kay, 1958). Harper, Kidd & Scratcherd (1959) have also shown that stimulation of the central end of the abdominal vagus in the cat causes secretion of acid and pepsin in the gastric juice.

The possibility that a humoral mechanism stimulates pepsin secretion

during the pyloric phase of secretion must also be considered in view of the long time lag, observed in the feeding experiments, before maximal secretion occurred. Although there is no conclusive evidence that pepsin secretion during the pyloric phase is controlled by other than nervous means yet the demonstration of a pepsin-stimulating hormone, gastrozymin (Blair, Harper & Lake, 1953) would, if confirmed, provide an alternative mechanism for pepsin secretion in the sheep.

Whether or not vagal stimulation plays a part in the secretory process the presence of vagal innervation appears to be important in ensuring maximum responsiveness of the gastric glands. This was shown by the diminished response of denervated pouches and conforms with the general conclusion in the literature that denervation reduces the secretory activity of the secretory glands to chemical stimuli.

The finding that volatile fatty acids, particularly acetic acid, had a pronounced effect on abomasal secretion was of interest because of the large amounts of these substances produced during rumen fermentation. Since it is well established that absorption of these acids takes place almost entirely from the reticulo-rumen and omasum (Barcroft *et al.* 1944) it is possible that their secretogogue effects are produced after absorption. On the other hand Popov (1932) and more recently Ash (1959) have shown that the addition of acetic acid directly to the abomasum stimulates gastric secretion and Russev (1959) has obtained similar results with propionic acid. Ash has also shown that rumen contents, when perfused through the abomasum, will increase secretion, and the possibility therefore exists that the VFAs only exert their effect when they reach the abomasum. Since the VFA levels in the abomasal ingesta are usually very low, stimulation of gastric secretion by this means may not be of very great significance. The lack of correlation between rumen VFA and abomasal secretion in the later stages of experiments, where there had been an initial correspondence between the two, calls for further enquiry but the decline in secretion may well have been due to inhibition brought about by the presence of highly acid gastric juice in the abomasum and duodenum.

SUMMARY

1. When sheep with innervated abomasal (gastric) pouches were allowed food *ad lib.*, secretion of gastric juice was continuous and showed no obvious relation to feeding. Fasting greatly reduced secretion and the ingestion of food was then followed by a secretory response in which maximum output of acid and pepsin occurred 1–1½ hr after feeding.

2. Smaller secretory responses with similar time courses were obtained from denervated abomasal pouches.

3. In a sheep with a Crocker–Markowitz cannula feeding after the

animal had been deprived of food resulted in an immediate increase in the flow of abomasal ingesta, which was maximal 1–1½ hr after feeding.

4. Distension of the pyloric region of the abomasum stimulated gastric secretion.

5. Sham-feeding and feeding of sheep in which the forestomachs were free from ingesta failed to stimulate abomasal secretion.

6. Insulin-induced hypoglycaemia produced a secretory response but this was mainly attributable to an increase in the rate of passage of ingesta to the abomasum brought about by reticulo-rumenal supermotility. Direct vagal effects on the gastric glands observed when the forestomachs were free from ingesta were confined mainly to stimulation of pepsin secretion.

7. Carbachol stimulated abomasal secretion which was not abolished by emptying the forestomachs.

8. The secretory response to a meal paralleled, in its early stages, the increase in concentration of volatile fatty acids in the rumen. Addition of food directly to the rumen also produced a secretory response, which paralleled the delayed increase in the concentration of volatile fatty acid observed under these conditions.

9. Acetic acid partially neutralized with NaOH was a potent stimulus to gastric secretion when added to the rumen. Propionic and butyric acids had smaller and less predictable effects.

10. It is concluded that the most important stimulus of gastric secretion in the sheep is the frequent passage of ingesta from the reticulo-rumen to the abomasum and that volatile fatty acids, particularly acetic acid, produced during rumen fermentation, may also play a part in the secretory response to feeding.

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POTASSIUM IN DIFFERENT LAYERS OF ISOLATED DIAPHRAGM

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Many measurements have been reported of the exchange of potassium between isolated tissues and the surrounding fluid. If the tissue is thick, then calculations lead to the conclusion that the exchange of potassium as measured in the tissue as a whole may be slower than the exchange for the individual fibres, owing to the retarding effect of intercellular diffusion (Harris & Burn, 1949; Keynes, 1954). When outward movement of ^{42}K is measured by placing the loaded muscle in flowing inactive saline, only the outer layer of cells is in an environment with negligible ^{42}K; most of the fibres are surrounded by interstitial fluid containing appreciable ^{42}K and the concentration gradient is correspondingly reduced.

There has been little experimental study of the effect of thickness on the exchange rate of tissues, and there is some evidence against the concept. Thus McLennan (1955) found only small differences at 20° C in the exchange for two muscles which were 3·5 and 0·5 mm thick, and concluded that intercellular diffusion was not in this case a rate-limiting factor. A different model has been proposed by Harris (1957) who considers that the exchange may be limited by intracellular diffusion.

If intercellular diffusion markedly affects the measured exchange rates, then it is important to establish this experimentally, so that corrections may be applied where necessary. One consequence of diffusion delays of this nature is that exchange will be non-uniform, the outer layers exchanging faster than the inner layers. It was thought possible that this might be demonstrated by slicing with a microtome.

Prolonged hypoxia produces depolarization in the deeper parts of an isolated diaphragm, while the superficial fibres show little change (Creese, Scholes & Whalen, 1958). There is also a loss of potassium, and it might be expected that hypoxia would lead to a depletion of potassium particularly from the deep tissues. This was also tested by slicing with a microtome.

Thick muscles (650 μ) have been employed, at 38° C. In these circum-

stances both the oxygen consumption and the potassium flux are high and it becomes easier to demonstrate differences between superficial and deep tissues.

METHODS

Animals. Albino rats weighing 160–180 *g* were used.

Saline. The composition was that used by Creese (1954) with 5·9 mM-K, at 38° C. In some cases a fraction of the potassium was present as ^{42}K. A gas mixture containing 5% (v/v) carbon dioxide and 95% oxygen was bubbled through the saline. For some experiments a mixture of 5% carbon dioxide, 5% oxygen and 90% nitrogen was used.

Handling of tissues. The left diaphragm was dissected in oxygenated saline and was attached by the rib to a simple holder made of glass rod. The muscles were soaked in saline contained in jacketed baths.

For the study of outward movement of ^{43}K the muscles, previously loaded with ^{42}K, were transferred to a jacketed bath containing an overflow tube, the arrangement resembling that used by Creese, Hashish & Scholes (1958), except that the bath was of a larger size and contained 10 ml. fluid. The muscle was suspended in the oxygenated saline so that the rib, which was uppermost, was held partly above the surface. A stream of warm saline flowing at a rate of 30 ml./min was led into the lower part of the bath; the saline flowed up around the muscle and was allowed to escape down the overflow tube. Doubling the flow rate did not increase the rate of exchange of ^{42}K.

Slicing methods. A solution containing gum acacia was frozen on the stage of a microtome and sectioned to provide a flat surface. The muscle was removed from the saline, wiped on a tile and carefully laid on the frozen surface, the pleural side being uppermost. The tissue was frozen, and the outer edges including the ribs were cut away for a distance of at least 2 mm and discarded. Horizontal sections of the remaining block of muscle were obtained, the microtome being set at 50 μ. The tissue was maintained frozen by a spray of carbon dioxide vapour. Muscles which were incorrectly positioned were discarded as described below.

Analysis of sections. There were usually about 15 sections or fragments of sections, and these were transferred to a pitted tile and the tissues were dried during the night at 105° C. In most cases the uppermost slice (pleural surface) and the middle slice were selected for analysis. The bottom slice was not used, as preliminary experiments showed that ^{42}K could sometimes be lost from that part of the muscle in immediate contact with the surface which had been frozen.

The dried fragments of tissue, weighing usually 0·2–0·4 mg, were conveyed by fine forceps to a small beaker and weighed to 0·01 mg. The tissues were then placed in tubes and dissolved by the addition of 0·1 ml. concentrated nitric acid and 0·1 ml. water, the tubes being placed in a boiling water-bath. After 20 min 1 ml. H_2O was added. The radioactivity in the solutions was compared by means of a well scintillation counter, with the usual corrections for background and decay. The activity was usually greater than 1000 counts.min^{-1}. The ^{42}K in slices was expressed as counts.min^{-1}.mg^{-1} (dry wt.).

For the results of Table 3 the fraction of ^{42}K remaining after soaking out for known times was required. The muscles were loaded by being placed in radioactive saline until the ratio (radioactivity associated with 1 μg K in muscle):(radioactivity associated with 1 μg K in saline) was unity. To obtain the ratio after the tissue had been placed in inactive flowing saline it was necessary to estimate the potassium content of the individual slices. This was usually about 2–8 μg K and was too small for convenient measurement by flame photometry. The potassium content was therefore estimated indirectly from the weight of the slice. The assumption that the total potassium concentration is the same at all depths in an oxygenated muscle is tested below (Table 1). In practice the upper slice (pleural surface), middle slice, and the remainder of the upper half of the muscle were weighed and dissolved

in nitric acid, and the radioactivity was measured. The contents of the tubes were then pooled and the total potassium was obtained by flame photometry. The potassium was allocated to the outer and middle slices on a weight basis, and the radioactivity associated with 1 μg K was calculated for the slices and for the tissue as a whole. The radioactivity associated with 1 μg K in the original saline was also obtained.

RESULTS

The effect of hypoxia

The effect of hypoxia in producing loss of potassium from muscles was confirmed. Diaphragms were soaked for 5 hr at 38° C in saline which was changed at intervals. With a gas mixture of 95 % (v/v) oxygen and 5 % carbon dioxide the potassium content was $361 \pm 7{\cdot}4$ μmole/g dry wt. (S.E. of 6 measurements). The value obtained by Creese (1954) was 374 μmole/g dry wt. Muscles gassed with 5 % oxygen, 5 % carbon dioxide and 90 % nitrogen had a potassium content of $156 \pm 6{\cdot}9$ μmole/g dry wt. (6 measurements). These muscles were pale and did not twitch when they were cut. The whole diaphragm was used in the analysis, including the edges.

From the electrical records obtained by Creese, Scholes & Whalen (1958) it was inferred that the loss of potassium found above was chiefly from the deeper layers of the muscle, and efforts were made to prove this by the microtome method. Early attempts were inconclusive, but it was found that the effect could be demonstrated providing the following conditions were satisfied. The net loss of potassium was over half, on a dry weight basis. The tissues were thick, at least 650μ when stretched. After soaking, the muscles were placed flat on the frozen surface for sectioning. The edges (which were probably better oxygenated than the other parts) were widely cut away. Muscles which appeared to be slanting or wrinkled when sectioned were discarded. In practice about two-thirds of all muscles which were soaked were considered unsatisfactory and only those in which sectioning appeared to be faultless were accepted for subsequent analysis. Consistent results were obtained by these procedures.

Table 1 gives results obtained on normal and hypoxic muscle. All the diaphragms were soaked for at least 5 hr in saline containing ^{42}K, with the gas mixture of 95 % oxygen and 5 % carbon dioxide, and in this time the ^{39}K and ^{42}K became uniformly mixed (Calkins, Taylor & Hastings, 1954; Creese, 1954). After this time the radioactivity of the slice was taken as a measure of the potassium content.

The left-hand column of Table 1 gives the ratio of the potassium content of the middle slice to that of the outer slice (pleural surface) for muscles which had been oxygenated normally. The mean value was close to unity, being $1{\cdot}01 \pm 0{\cdot}044$ (S.E. of 11); the range was large, from 0·81 to 1·22.

Muscles which had been treated for 5 hr in the above manner were subsequently made hypoxic by the use of a mixture containing 5 % oxygen for a further 5 hr. It was found that the ratio was always lower than the control values (Table 1), being $0{\cdot}44 \pm 0{\cdot}044$ (S.E. of 10). This was taken to indicate a diminished potassium content of the middle slice.

Hypoxia produces various artifacts in the course of outward movement of ^{42}K. In Fig. 1 the muscle, previously loaded for 4 hr with ^{42}K, has been placed in inactive flowing saline, the apparatus being similar to that used by Creese (1954) except that the oxygen jet which is normally directed on the muscle was withdrawn. In these circumstances the oxygenation is

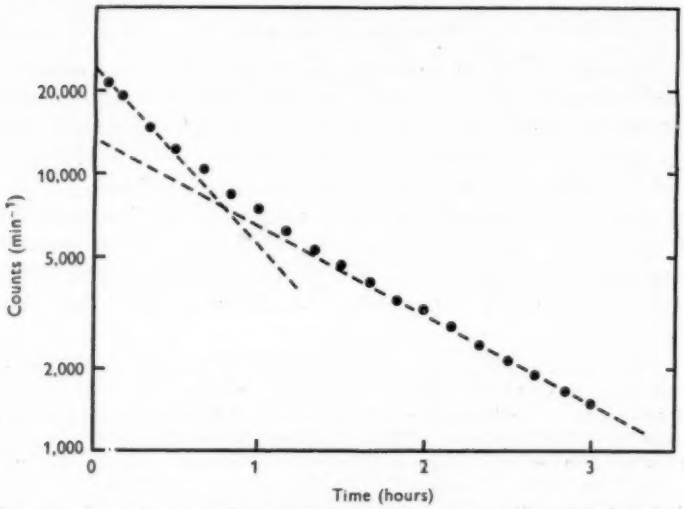

Fig. 1. Outward movement of ^{42}K from diaphragm which received insufficient oxygen from the start of the washout. There is net loss of potassium (see text). The ordinate gives the ^{42}K remaining in the tissue on a semi-log. scale. The curve could be described as the sum of two exponential terms.

TABLE 1. The effect of hypoxia on the potassium in the middle slice of the muscle compared with that in the outer slice. The results are expressed as ratios, calculated on a dry-weight basis

	Oxygenated (95 % O_2, 5 % CO_2)	Hypoxic (5 % O_2, 5 % CO_2, 90 % N_2)
	0·89	0·45
	0·95	0·64
	0·99	0·39
	1·19	0·29
	0·81	0·61
	1·09	0·46
	0·93	0·23
	1·15	0·31
	1·22	0·53
	0·82	0·53
	1·08	—
Mean	1·01	0·44
S.E.	0·044	0·044

The hypoxic muscles show diminished [K] in the middle slice ($P < 0·01$). The potassium content of the slices was measured by a method involving ^{42}K.

insufficient and there is a net loss of potassium (Creese, 1954). If the curve relating radioactivity to time is plotted on semi-logarithmic paper, there is now a pronounced kink in the curve (Fig. 1). Similar results were obtained in eight experiments. These curves could be expressed as the sum of two exponentials. Figure 1 may be contrasted with the results obtained when full oxygenation is employed with no net loss of potassium, which gives a smooth curve with only a small change in the slope as time proceeds (see Fig. 8, Creese 1954).

TABLE 2. ^{42}K in slices after inward movement for 20 min (counts.min^{-1}.mg^{-1} dry wt.)

Outer slice	Middle slice	Ratio middle:outer
1395	745	0·61
1400	987	0·70
1429	1210	0·80
1675	1063	0·52
2556	1933	0·69
2506	2313	0·87
1888	1187	0·44
1712	1343	0·74
3410	2660	0·78
1718	1327	0·77
	Mean	0·69
	S.E.	0·04

The uptake of the middle slice after 20 min is less than that of the outer slice ($P < 0·01$).

Inward movement of ^{42}K

Muscles were soaked for 2 hr in saline gassed with 95 % oxygen and 5 % carbon dioxide, and were then transferred for 20 min to saline whose potassium was partly in the form of ^{42}K. The muscles were removed and sliced with the microtome, and tissues which had been sectioned satisfactorily were analysed for radioactivity. Table 2 gives the ratio of activity in middle slice to that in the outer slice. All muscles which were counted gave values less than unity, the mean being $0·69 \pm 0·040$ (S.E. of 10, $P < 0·01$). This is taken as evidence that uptake is non-uniform, the fibres in the depths of the tissue showing delayed exchange. Some 2 % of the total potassium is extracellular (Creese, 1954); no correction has been made to allow for this factor.

Outward movement of ^{42}K

Diaphragms were soaked for 4 hr in saline containing ^{42}K, so that ^{39}K and ^{42}K became completely mixed. The tissues were gassed with 95 % oxygen and 5 % carbon dioxide. The muscles were then placed for known periods in inactive flowing saline and were subsequently sectioned and analysed. Table 3 shows the fractions of ^{42}K remaining in the outer slice,

the middle slice, and in the muscle as a whole. In the case of the outer and middle slices it was not possible to estimate the potassium content directly, and an indirect method was used as described above.

The rate constant for the whole muscle over the first 30 min was 1·0 hr^{-1}, and f, the fraction of ^{42}K remaining in the whole muscle, was 0·607. Previous results with somewhat thinner diaphragms gave 1·2 hr^{-1} (Creese, Neil & Stephenson, 1956). It can be seen from Table 3 that in each of 14 muscles the fraction remaining in the middle slice is greater than that of

TABLE 3. Outward movement of ^{42}K

Time	Middle slice	Outer slice	Whole muscle	Apparent rate coefficient (hr^{-1})
After 30 min	0·827	0·397	0·638	
	0·713	0·377	0·588	
	0·590	0·361	0·530	
	0·797	0·498	0·674	
	0·750	0·360	0·641	
	0·712	0·456	0·574	
Means	0·732	0·408	0·607	1·00
After 60 min	0·322	0·215	0·309	
	0·522	0·182	0·385	
	0·465	0·263	0·354	
	0·341	0·230	0·307	
Means	0·415	0·222	0·339	1·08
After 90 min	0·352	0·222	0·291	
	0·184	0·060	0·176	
	0·258	0·153	0·214	
	0·236	0·170	0·209	
Means	0·258	0·151	0·222	1·00

Results are expressed as the fraction of ^{42}K remaining in the middle and outer slices and in the tissue as a whole after the muscle, previously loaded with ^{42}K, had been placed in inactive flowing saline. The last column gives the rate coefficient for the whole muscle. The fraction was unity at the start of the washout.

the muscle as a whole, while the fraction in the outer slice is smaller. This supports the view that the ^{42}K in the superficial layers is lost comparatively quickly, while that in the deeper fibres lags behind. The over-all average ratio of the fractions remaining in the inner and outer slices is 1·81. It is possible that this may be an under-estimate and that the extreme outer fibres exchange even more rapidly (see below).

Theoretical distribution of ^{42}K across the muscle

Figure 2 represents a section across the muscle of thickness 650 μ after 30 min of outward movement of ^{42}K. The ordinate gives the fraction of ^{42}K which remains after this time, calculated by the method of Harris & Burn (1949) as described below. At zero time the value was unity and after 30 min the distribution becomes U-shaped and symmetrical, convex up-

wards, the fraction which remains at the edge of the muscle being smaller than that in the centre portion. The dotted line gives the mean value, which from Table 3 is 0·607.

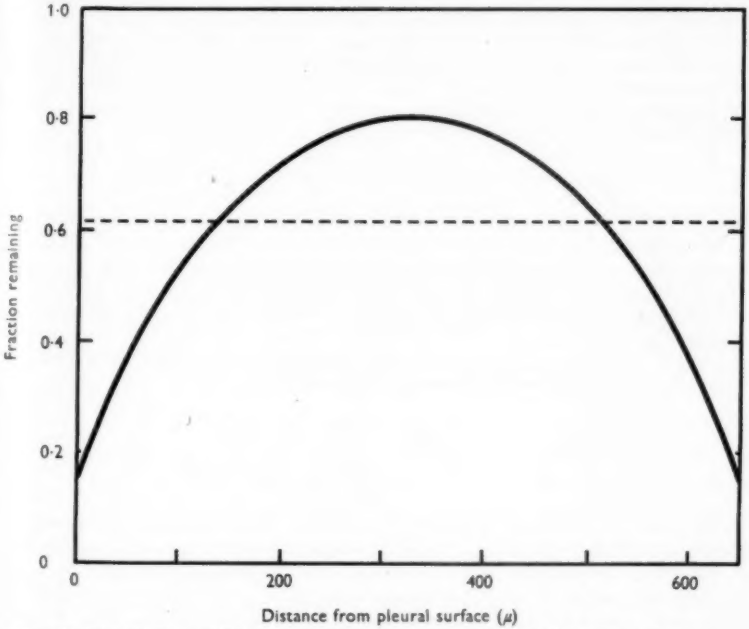

Fig. 2. Distribution of ^{42}K across a thick diaphragm (650 μ) after outward movement for 30 min, obtained from equations of Harris & Burn (1949). Ordinate gives fraction remaining; the initial fraction was unity. Abscissa gives distance from pleural surface. The dotted line gives the mean for the whole muscle.

The combination of exponential kinetics across the cell membrane plus intercellular diffusion has been treated by Harris & Burn (1949). The measured exchange of the outer fibres is faster than that of the deep fibres because the cells are in different environments: the content of ^{42}K in the intercellular fluid differs from that of the external saline because of diffusion lags.

In calculations of the distribution of ^{42}K across the muscle the nomenclature of the above authors has been retained, except that k is here used in place of k_3 and their term D/λ^2 has been replaced by aD' where a is the fraction by volume of intercellular space and D' is the apparent diffusion coefficient of potassium in the interspaces (Carey & Conway, 1954; Keynes, 1954). With this adjustment it can be shown that there is agreement between Harris & Burn (1949) and Keynes (1954) as regards the corrections to be applied to the experimental results to allow for the effect of intercellular diffusion (Creese *et al.* 1956). In the following treatment mean values are considered for the results obtained after outward movement of ^{42}K for 30 min (Table 3), and other values for diaphragm are those used by Creese *et al.* (1956). Approximations have been used when the precision of the data did not justify more elaborate treatment.

After outward movement of ^{42}K for 30 min the fraction of ^{42}K remaining is 0·607. This equals $\exp(-k't)$ where k, the apparent rate constant for the whole muscle, is 1·00 hr^{-1}. The constant r is obtained as follows:

$$b\,k_1 = r\,\pi^2\,D/\lambda^2\,l^2. \tag{1}$$

Here b, the fractional volume of the fibres, is 0·55; $k_1 = 25\,k$ where k is the true rate constant for outward movement of ^{42}K and 25 is the ratio of internal to external potassium: l, the muscle thickness, is 0·065 cm; D/λ^2 is aD' or $0{\cdot}28 \times 5{\cdot}2 \times 10^{-6}$ $cm^2.sec^{-1}$. Then $r = 4040\,k$. Except for l, the muscle thickness, these values have been taken from Creese (1954).

The fraction f of radioactivity remaining after time t may be expressed by equation (8b) of Harris & Burn (1949) in the following form:

$$f = \frac{8}{\pi^2}\left[0{\cdot}121\exp(-kt) + \exp\left(\frac{-kt}{1+r}\right) + 0{\cdot}111\exp\left(\frac{-9\,kt}{9+r}\right)\right]. \tag{2}$$

Now f is 0·607 and t is 1800 sec. By trial and error, r is found to be 4·0, so that kt is 1·78 and k is 3·56 hr^{-1}. This indicates that k'/k is about 0·28 for this thick muscle. The fraction remaining in the outermost portion is thus $\exp(-kt)$ or 0·169, as shown in Fig. 2.

It can be shown that when r approaches zero then the expression in equation (2) approaches $\exp(-kt)$, as given by a single fibre or a thin muscle for which the diffusion lag is negligible.

The fraction remaining in the centre of the muscle was obtained by the use of equation (5) of Harris & Burn (1949) in the following simplified form:

$$f = \frac{4}{\pi}\sum_{0}^{\infty}\frac{1}{2n+1}\,(-1)^n\exp(-mkt), \tag{3}$$

where

$$m = \frac{(2n+1)^2}{r+(2n+1)^2}.$$

For the values after 30 min, k is 3·5 hr^{-1}. The fraction is given by the sum of a slowly converging oscillating series, and for 13 terms the value is 0·802. This has been used in Fig. 2.

Thus in Fig. 2 we have the calculated value at the outer edge of the muscle, the value at the centre, and also the mean. The curve was obtained by setting up an arbitrary U-shaped function which satisfied the above requirements. This function is

$$f = f_0 - cz^2 - dz^3, \tag{4}$$

where f is the fraction remaining after washout for 30 min at a distance z from the centre, f_0 is the value at the centre (= 0·802), and z is expressed for convenience in arbitrary units such that there are 3 in each half thickness. With a total thickness of 650 μ, each arbitrary unit of distance equals 108·3μ. The constants c and d are 0·0450 and 0·00844, respectively. A somewhat similar procedure for obtaining the radioactivity at various depths in the muscle has been used by Harris & Burn (1949).

Comparison of results with theory

From the theoretical distribution shown in Fig. 2 it is expected that the values of the outer edge and at the centre should differ appreciably from the mean for the whole muscle. From Table 3 the differences found by experiment are smaller than those predicted.

Comparison is rendered difficult because the depth of the slices is not known with precision. Thus the 'centre slice' was probably within the middle 100 μ (50 μ either side of the mid line), and from Fig. 2 the average value is 0·796. The experimental value is 0·732. In the case of the 'outer slice' the first section was accepted if it appeared to be over 25 μ thick; small outer snippets less than 25 μ thick were discarded and the first full slice of 50 μ

was taken. This policy would yield an estimate of the average value of the outer 75 μ. In Fig. 2 the outer 75 μ averages 0·322, as compared with the experimental value of 0·408.

The rate constant of the individual fibres may be calculated from the mean value by means of equation (2), or obtained from the experimental results. From Table 3, f_0 is 0·732 at the centre when z is zero. Also f is 0·408 for the 'outer slice', and if this may be considered as 75 μ thick we may take z as approximately 37 μ from the outer edge, or 2·65 units from the centre. Two points on the curve are known, and there is an additional requirement that the mean is 0·607. An arbitrary function can be set up similar to that in equation (4) which fulfils these conditions. In this case f_0 is 0·732, c is 0·0175 and d is 0·0107. Then the value of f at the outer edge, where z is 3 units, is 0·285. The rate constant k for the fibres is hence $\{-(1/t)\ln f\}$ hr^{-1} or 2·51 hr^{-1}.

TABLE 4. Expected and experimental results after outward movement of ^{42}K for 30 min

	Expected	Experimental
Fraction remaining in whole muscle	—	0·607
Fraction remaining in centre slice	0·796	0·732
Fraction remaining in outer slice	0·322	0·408
Apparent rate coefficient for whole muscle (hr^{-1})	—	1·00
Rate coefficient of fibres (hr^{-1})	3·5	2·5

The values for the expected results have been obtained by use of the equations derived by Harris & Burn (1949).

Table 4 summarizes the findings for 30 min. It is evident that the experimental differences between layers are somewhat less than those anticipated from the combination of diffusion plus exponential kinetics. The theory over-corrects the experimental findings. Taking the values for the rate constant of the fibres, the ratio of the experimental value to the calculated figure is 0·70. If the 'outer slice' is taken as giving the value for the outer 50 μ, then the ratio is 0·60.

DISCUSSION

Following Hill's paper (1928), the effect of intercellular diffusion on the exchange between an isolated tissue and the surrounding fluid has been discussed repeatedly, with conflicting findings in the case of ion exchange. It has not been easy to devise methods which gave consistent results, and initial failures were obtained in the present investigation. Any method of slicing which is employed should first be used to demonstrate the effects of anoxia; if a difference can be shown between inner and outer parts of the muscle, then the method can be used with reasonable confidence in other situations. It should be emphasized that it was only possible to obtain consistent results with thick muscles of 650 μ; the findings were equivocal with thinner tissues.

Though qualitative differences between the outer and inner slices were demonstrated the quantitative aspect was less satisfactory. After every allowance had been made it was found that the retarding effect of diffusion

was only some 60–70% of that which was expected from the equations given by Harris & Burn (1949) and Keynes (1954). There are of course many uncertainties in these estimations and these include errors in the measurement of the apparent diffusion coefficient of potassium and other constants, the simplifications introduced into the model whereby the muscle is treated as a flat homogeneous slab, and the necessary mathematical approximations. As Keynes (1954) states, the equations are likely to over-correct for the effects of diffusion and produce an exaggerated result. Thus Creese *et al.* (1956), when studying potassium exchange in diaphragm, considered that to obtain the true rate constant of the fibres from the measured rate constant of the whole tissue it was necessary to multiply the latter value by a factor of about three. The present findings imply that a factor of two would be more appropriate, and the calculated values for the potassium flux and also the amount of potassium lost per twitch (Creese, Hashish & Scholes, 1958) may be too high.

Diaphragm muscle must be regarded as a 'thick' tissue in which corrections are required in kinetic studies of potassium movements. As regards isolated amphibian muscle, Keynes (1954) has given reasons for regarding ion exchange in the toe muscle as unaffected by extracellular diffusion, at room temperature. Conway (1957) has shown that ion exchange in frog sartorius muscle in certain circumstances at 0–3° C is such that the thickness does not affect the process, and the preparation can be treated for this purpose as though single fibres were being used. There is some doubt regarding frog sartorius muscle at room temperature. Keynes (1954) calculated that diffusion delayed exchange of potassium by a factor of about three when the muscle was exposed to the solution on one side, and the correction was very nearly as large in the case of muscles immersed on both sides in unstirred solution. If the solution is well stirred then the correction should be appreciably smaller. Taking the example in which the measured exchange rate is 0·088 hr^{-1} at 16° C (Keynes, 1954) and the other factors including the diffusion coefficient of potassium through the interspaces are as for the single-sided case, then it can be shown that M', the apparent flux, is 4·9 $pmole.cm^{-2}.sec^{-1}$ and the true flux obtained by Keynes's method is 6·5 $pmole.cm^{-2}.sec^{-1}$. The diffusion factor is then about 1·3, and this might not be detected by the slicing methods used above. Harris & Steinbach (1956) sectioned muscles into three longitudinal slices after uptake of ^{42}K for various times and were unable to detect differences in specific activity between the inner third and the outer portions.

It is possible by vigorous oxygenation to maintain the potassium content of diaphragm muscle for many hours at values found *in vivo* (Creese, 1954). Many isolated mammalian preparations are used with only a trickle

of oxygen, and it is not known whether aeration is sufficient in these conditions. Anoxia in diaphragm muscle leads to loss of potassium and depolarization of inner fibres (Creese, Scholes & Whalen, 1958), as well as the changes in distribution of potassium shown in Table 1. With inadequate oxygenation there also appears a prominent kink in the washout curve when the activity remaining in the tissue is plotted against time on semi-logarithmic paper. The radioactivity remaining in the muscle could now be described in terms of the sum of two exponential processes, and this is probably due to the differences between normal cells in the superficial part of the muscle and the anoxic depolarized central fibres. When curves of this nature are met elsewhere it is necessary to ensure that they are not artifacts associated with a net loss of potassium.

SUMMARY

1. Rat diaphragms (650 μ thick) were soaked in saline and the flat muscles were frozen and sliced horizontally with a microtome. After normal oxygenation the potassium contents of deep and superficial layers were similar, as measured on a dry weight basis by a method involving the use of ^{42}K.

2. After prolonged hypoxia, obtained by reducing the oxygen in the gas mixture to 5 %, the inner fibres were depleted of potassium, the ratio middle : outer slice being 0·44 (mean of 10).

3. In oxygenated saline the exchange of ^{42}K was non-uniform, being slower in the deeper fibres. After uptake for 20 min the ^{42}K in the outer slice exceeded that in the middle slice. After outward movement into inactive saline for 30–90 min the superficial fibres lost more ^{42}K than the deeper fibres, the ratio middle : outer slice being 1·81 (mean of 14).

4. The kinetic results are consistent with the view that the exchange rates of thick tissues may be diminished by intercellular diffusion. The differences found between superficial and deep layers were less than those predicted by the assumption of diffusion kinetics plus exponential exchange.

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EFFECTS OF THYROXINE ON THE SYNTHESIS OF CHOLESTEROL AND FATTY ACIDS BY CELL-FREE FRACTIONS OF RAT LIVER

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We have shown elsewhere (Fletcher & Myant, 1958) that the synthesis of cholesterol from acetate by rat liver slices is increased if the rat is given injections of thyroxine before the liver is removed. If mevalonic acid, instead of acetate, is used as the precursor of cholesterol, this effect is absent or only just detectable. When the daily dose of thyroxine is 20 μg or more, synthesis of fatty acids by the liver slices is depressed. It was suggested that treatment with thyroxine stimulates cholesterol synthesis by acting upon a rate-limiting step between acetate and mevalonic acid, and that the depression of fatty acid synthesis might be due to a deficiency of ATP.

In this paper we describe experiments designed to test whether these effects of thyroxine can be localized in one or other of the fractions of the liver cell. Cholesterol (Bucher & McGarrahan, 1956) and fatty acids (Langdon, 1955; Seubert, Greull & Lynen, 1957) have been shown to be synthesized *in vitro* by homogenates of mammalian liver from which the heavier subcellular particles have been removed. We have therefore studied cholesterol and fatty acid synthesis in such preparations, obtained from the livers of thyroxine-treated and normal rats.

METHODS

Animals. The rats were albino males of an inbred strain, weighing 120–200 g. The treated animals were given sodium-L-thyroxine (20–50 μg in 0·2 ml. water) daily for 9 days by intraperitoneal injection under light ether anaesthesia. The solutions of thyroxine were made by dissolving the required amount in a small quantity of water with the addition of one drop of 20% NaOH, and then diluting to 100 ml. with water. Control animals were similarly injected with 0·2 ml. of an alkaline solution containing no thyroxine. During treatment with 20 or 30 μg of thyroxine per day, the weight gain and food consumption of the treated rats did not differ significantly from that of the controls. When the daily dose of thyroxine was 50 μg, the treated animals gained less weight than the controls.

Preparation of tissue. At the end of the treatment, the animals were killed by breaking their necks. The livers were removed as rapidly as possible and placed on ice. Homogenates were then prepared from the pooled livers of each group of treated and control rats,

according to the technique of Bucher & McGarrahan (1956) as modified by Popják, Gosselin, Gore & Gould (1958), in buffer of the following composition: 0·1 M potassium phosphate buffer at pH 7·4; 0·005 M magnesium chloride; 0·03 M nicotinamide; 0·003 M glutathione. Nuclei and tissue debris were removed from the homogenate by centrifuging at 2000 *g* for 25 min. Mitochondria were removed from the supernatant of the first spinning by centrifugation at 10,000 *g* for 20 min. The supernatant from this second spinning is designated S_{10}. To separate the microsomes, S_{10} was spun in a Spinco preparative ultracentrifuge at 104,000 *g* for 1 hr. The supernatant fraction (S_{104}) was decanted from the pellet (*M*) containing microsomes and glycogen. Pellet *M* was then suspended in a volume of buffer equal to one quarter of the volume of S_{104}, by gently stirring with a small glass homogenizer, and was again spun at 104,000 *g* for a further 60 min. S_{104} was also centrifuged a second time to throw down any residual microsomes. After the washed microsomes had been spun, fraction *M* was suspended in a volume of buffer equal to 1/10 the original volume of S_{104}. All these operations were carried out at 0° C. It has been shown (Popják *et al.* 1958) that fraction *M* prepared by this method contains no mitochondrial cytochrome and that only cytochrome b_5 is present.

Incubation of tissues. The following abbreviations are used in this paper: DPN and DPNH, diphosphopyridine nucleotide in oxidized and reduced form; TPN and TPNH, triphosphopyridine nucleotide in oxidized and reduced form; ATP, adenosine triphosphate; EDTA, ethylenediaminetetraacetate. All incubations were carried out in duplicate in a standard medium containing the following substances, other substances being added in addition to these: DPNH, 1 μmole; TPN, 0·5 μmole; glutathione, 20 μmole; EDTA, 3·5 μmole. [1–^{14}C]-sodium acetate (20 μmole containing 5 μc dissolved in 0·25 ml. buffer) or [2–^{14}C]-mevalonic acid (5 μmole containing 1 μc in 0·05 ml. buffer) was added as the precursor for cholesterol and fatty acid synthesis. Either 2·5 ml. of S_{10}, or 2·5 ml. of S_{104} plus 0·25 ml. of microsome suspension (*M*), was added to each incubation flask (vol. 50 ml.) and the volume made up to 3·5 ml. by the addition of buffer. Incubations were carried out with a gas phase of air, in a constant-temperature bath at 37° C for 2 hr, with constant shaking.

Extraction of cholesterol and fatty acids. After incubation, 1 ml. of saturated NaOH, 3 ml. of ethanol and 2 mg of cholesterol in ethanol were added to the flasks and the contents saponified by heating at about 70° C for 2 hr. Cholesterol was extracted from the alkaline hydrolysate with light petroleum ether and precipitated as the digitonide. The digitonide was filtered on to weighed perforated planchettes containing a disk of filter paper, washed and dried. For extraction of the fatty acids, the residue of the original saponification mixture, plus the washings from the petroleum ether extracts, were acidified and extracted twice with petroleum ether. The petroleum ether was washed once with 0·1% acetic acid and twice with water, and evaporated to dryness, the residue being dissolved in chloroform. The fatty acids in the chloroform were titrated with 0·1 N-NaOH to a pink colour with phenolphthalein. The soaps were evaporated to dryness, dissolved in a little water, and transferred to weighed planchettes containing lens paper. The planchettes were then dried and weighed.

Measurement of radioactivity. Radioactivity was measured with a mica window G–M counter (EHM 2/S, G.E.C. Research Laboratories). All counting rates were corrected to the rate at 'infinite thickness' by means of standard self-absorption curves.

RESULTS

Synthesis of cholesterol and fatty acids in S_{10} fraction

Our first aim in these experiments was to see whether the effects of thyroxine treatment on the synthesis of lipid by liver slices could also be

observed in cell-free fractions of liver. Groups of rats were given daily injections of 20, 30 or 50 μg of thyroxine. Synthesis of cholesterol and fatty acids was then compared in the S_{10} fractions prepared from each group of treated and control rats. The results of each experiment were expressed as the ratio of the counting rate at 'infinite thickness' of cholesterol digitonide or soaps of the treated, to that of the control S_{10} fraction.

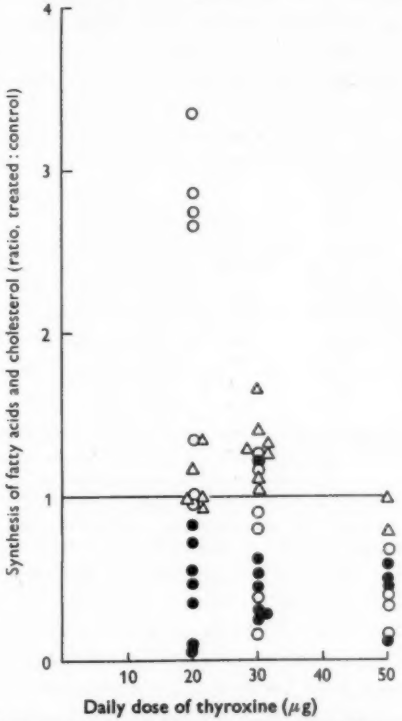

Fig. 1. Effect of thyroxine on synthesis of cholesterol from acetate (○) and mevalonic acid (△), and of fatty acids (●), in S_{10} fraction of liver. Each point represents the results obtained from one experiment in which the values from duplicate incubations of tissue from the pooled livers of six treated rats were compared with the corresponding values from six control rats. Points show ratio of treated to control values.

The effect of thyroxine on the synthesis of cholesterol from acetate varied with the dose given. With 20 μg of thyroxine, there was an increase in synthesis (Fig. 1) comparable with that in slices. With 30 and 50 μg there was usually a moderate depression of synthesis. When mevalonic acid was used as the labelled precursor, doses of 20 or 50 μg of thyroxine

had little or no effect on cholesterol synthesis; with 30 μg, synthesis was slightly increased (Fig. 1).

Synthesis of fatty acids was depressed after treatment with thyroxine at all three dose levels (Fig. 1).

Synthesis of cholesterol and fatty acids in fractions obtained from S_{10}

The results obtained with S_{10} show that effects of thyroxine treatment can be observed in fractions of liver cells from which nuclei and mitochondria have been removed. The next step was to see whether these effects could be localized either in the particles sedimenting at 104,000 *g*

TABLE 1. Effect upon cholesterol and fatty acid synthesis from acetate of recombining S_{104} and *M* fractions from treated and control livers and of removing and adding glycogen

Daily dose of thyroxine (μg)		Fractions recombined						
		$S_{104}(C)+M(C)$	$S_{104}(T)+M(T)$	$S_{104}(T)+M(C)$	$S_{104}(C)+M(T)$	$S_{104}(C)+\bar{M}(C)$	$S_{104}(T)+\bar{M}(T)+G(C)$	$S_{104}(T)+M(T)+G_{ex}$
20	Cholesterol	2607	9001	4520	4705	—	—	—
	Fatty acids	2925	1412	3560	1030	—	—	—
30	Cholesterol	3588	382	3620	285	866	2615	3830
	Fatty acids	2485	554	2574	524	1250	1096	1723
50	Cholesterol	2712	662	2994	941	—	—	—
	Fatty acids	1893	816	2092	776	—	—	—

$S_{104}(C)$ and $S_{104}(T)$, supernatant fractions obtained after spinning S_{10} from control and treated rats at 104,000 *g*; $M(C)$ and $M(T)$, sediments from S_{10} spun at 104,000 *g*; $\bar{M}(C)$, fraction obtained by removing glycogen from $M(C)$; $G(C)$, glycogen from $M(C)$; G_{ex}, glycogen prepared from normal rat liver by Somogyi's method (Somogyi, 1934).

Each value is the average counting rate (counts/min) at 'infinite thickness' of the digitonide or soap obtained from paired incubation flasks. The values in each pair from a given pooled preparation usually differed from each other by less than 10%.

(*M* fraction) or in the supernatant (S_{104} fraction). *M* and S_{104} were obtained from the livers of thyroxine-treated and control rats. The two fractions from the treated rats ($M(T)$ and $S_{104}(T)$) were then recombined with the fractions from the control rats ($M(C)$ and $S_{104}(C)$) as shown in Table 1. These recombinations were tested for their ability to synthesize cholesterol and fatty acids from acetate.

Table 1 shows some of the results obtained with livers from rats given daily injections of 20, 30 or 50 μg of thyroxine. The increase in cholesterol synthesis occurring after 20 μg of thyroxine seems to be due to an effect upon both fractions, since both the recombinations which contained a fraction from the treated livers ($S_{104}(T)+M(C)$ and $S_{104}(C)+M(T)$) synthesized more cholesterol than the recombination in which both fractions were derived from the control livers ($S_{104}(C)+M(C)$). When there was a decrease in cholesterol synthesis the effect was confined to the *M* fraction,

since the recombination $S_{104}(C)+M(T)$ synthesized less cholesterol than did the control recombination, whereas $S_{104}(T)+M(C)$ synthesized a normal amount. Similarly, the decrease in fatty acid synthesis, observed at all three dose levels, was due to an effect on the *M* fraction of the treated livers.

Fractionation of the microsome pellet

The finding that the inhibitory action of thyroxine on cholesterol and fatty acid synthesis is due to an effect on the *M* fraction led us to examine this fraction in more detail. When the S_{10} from a normal rat has been spun at 104,000 *g*, the contents of the tube are separated into several layers, as described by Popják *et al.* (1958). A thin layer of fat floats on top of a clear red solution occupying about 4/5 of the tube; this is the S_{104}. Beneath this is a pellet consisting of two sharply differentiated layers. The upper one is opaque and red and contains the microsomes; the lower one is a clear pale yellow and consists mainly of glycogen and a few heavy microsomes. In preparations from the livers of thyroxine-treated rats, the glycogen layer was found to be much diminished or absent, the amount present being roughly dependent on the dose of thyroxine given. After doses of 30 or more μg/day, glycogen could seldom be detected by the naked eye.

This suggested that the inhibitory action of thyroxine might be due to a reduction in the amount of glycogen in the preparations tested for cholesterol and fatty acid synthesis *in vitro*. In order to test this possibility, synthesis from acetate was studied in control preparations from which the glycogen was removed, and in treated preparations, obtained from rats given 30 μg of thyroxine per day, to which glycogen was added *in vitro*. By gently shaking the *M* fraction with a little buffer, the layer of microsomes could be separated almost completely from the glycogen. Recombinations could then be made containing either $S_{104}(C)$ with the control microsome pellet from which the glycogen had been removed ($\bar{M}(C)$), or $S_{104}(T)$ with $M(T)$ to which exogenous glycogen or the glycogen from the control preparations had been added. Removal of the glycogen from the control preparations depressed synthesis of cholesterol and fatty acids, though not to the low level observed in preparations made by recombining $S_{104}(T)$ with $M(T)$ (Table 1). When the glycogen from the control liver preparations was added to the recombination containing $S_{104}(T)$ and $M(T)$, synthesis of cholesterol and fatty acids was increased, though it was not fully restored. When exogenous glycogen (10 mg) was added, synthesis of cholesterol was usually restored to the normal level, and synthesis of fatty acids was increased to a greater extent than with glycogen from the $M(C)$ fraction (Table 1).

DISCUSSION

These results show that, under certain conditions, our findings with liver slices can be reproduced in the cell-free extracts described in this paper. With low doses of thyroxine (20 μg/day), there is a two- to three-fold rise in cholesterol synthesis from acetate. With higher doses, however, synthesis is usually reduced. When mevalonic acid is used as substrate for cholesterol the results observed after 20 and 30 μg of thyroxine/day are similar to those obtained with slices—there is either no change, or a small increase in synthesis. At all dose levels the effect of thyroxine treatment on the synthesis of fatty acids was similar to that observed in slices.

An important difference between slices and the cell-free preparations used here is the absence of mitochondria from the latter. Mitochondria, by bringing about oxidative phosphorylation, provide most of the ATP within a normal liver cell. Since mitochondria were removed from our preparations, the ATP available for synthesis must have been limited to that present in the soluble fraction, plus that generated by glycolysis. ATP is required in considerable amounts for the synthesis both of cholesterol and of fatty acids. For the synthesis of cholesterol from acetate, 1 mole of ATP is required for the formation of each of 18 moles of acetyl CoA, and a further 18 moles of ATP are required for the phosphorylation of the 6 moles of mevalonic acid formed from the acetyl CoA (Cornforth, 1959). The steps by which fatty acids are synthesized in mammalian liver are not fully understood, but at least 9 (Lynen, 1959), and possibly up to 18 (Titchener, Gibson & Wakil, 1958) moles of ATP are required for the synthesis of one mole of fatty acid containing 18 carbon atoms per molecule.

Since such large quantities of ATP are required for the formation of cholesterol and fatty acids, homogenates from which the mitochondria have been removed might be expected to have little capacity for synthesis. However, Bucher & McGarrahan (1956) concluded that in their preparations glycolysis could supply all the ATP required for cholesterol synthesis, and in our preparations from control rats, synthesis of fatty acids and cholesterol was comparable with the amounts formed in slices. In the homogenates from treated rats, on the other hand, glycogen, which is a substrate for glycolysis, was reduced or absent. In these preparations, therefore, the supply of ATP may have been limited by a shortage of glycolytic, as well as of oxidative, phosphorylation. This suggests that when there was a depression of cholesterol and fatty acid synthesis from acetate (Fig. 1 and Table 1) it was due to a reduction in the supply of ATP generated by the break-down of glycogen. This is supported by the finding that synthesis of both substances was increased by adding glycogen to the

treated preparation and was depressed by removing glycogen from the controls (Table 1). It must be pointed out, however, that fatty acid synthesis could never be restored to the control level by addition of glycogen.

With doses of thyroxine sufficient to depress the synthesis of cholesterol and fatty acids from acetate, there may be no depression of cholesterol synthesis from mevalonic acid (Fig. 1). Since ATP is required for the formation of cholesterol from mevalonic acid, this may appear to conflict with our suggestion that the low synthesis from acetate is due to a shortage of the glycolytic source of ATP. However, there is no reason to assume that a fall in the concentration of ATP would have the same effect on synthesis from mevalonic acid as from acetate. If, for example, the enzymes phosphorylating mevalonic acid have a higher affinity for ATP than those concerned in the activation of acetate in the presence of ATP, a fall in the concentration of ATP might reduce synthesis from acetate before it had any effect on synthesis from mevalonic acid.

A deficiency of glycogen might depress synthesis by diminishing the supply, not only of ATP, but also of TPNH and DPNH. Since both these co-enzymes are probably required in their reduced forms for the synthesis of cholesterol (Bucher & McGarrahan, 1956; Popják *et al.* 1958) and fatty acids (Langdon, 1955), some mechanism for keeping them reduced may be necessary if synthesis is to take place. In the liver, an important reaction leading to the reduction of TPN to TPNH is the oxidation of glucose-6-phosphate. This substance is formed either from glucose or as an intermediate in the break-down of glycogen. DPN is also reduced by the oxidation of a substance derived from glycogen (3-phosphoglyceraldehyde). Shortage of glycogen could, therefore, cause a diminution in the amounts of TPNH and DPNH formed in the cell-free preparations.

In slices from thyroxine-treated rats there is a depression of fatty acid synthesis (Fletcher & Myant, 1958). It is generally believed that most of the ATP required for syntheses in the whole cell is generated by oxidative phosphorylation in the mitochondria. If this is so, a shortage of glycogen should not affect the supply of ATP needed for fatty acid synthesis in slices. Possibly the fall in glycogen content affects fatty acid synthesis in the slices by interfering with the supply of reduced co-enzymes. The well-known action of thyroxine in inhibiting oxidative phosphorylation in the mitochondria (Lardy & Feldott, 1951; Martius & Hess, 1951; Niemeyer, Crane, Kennedy & Lipmann, 1951) is unlikely to be the cause of the depression of fatty acid synthesis, since this inhibition has been observed only after doses of thyroxine many times larger than those we have used.

A deficiency of glycogen may well be the main factor causing a fall in cholesterol and fatty acid synthesis in the cell-free preparations. It must

be admitted, however, that it cannot, by itself, explain the rise in cholesterol synthesis from acetate that occurs in slices with doses of thyroxine varying from 10 to 500 μg/day (Fletcher & Myant, 1958); nor can it explain the rise observed in the cell-free preparations after 20 μg of thyroxine.

SUMMARY

1. The synthesis of cholesterol and fatty acids was measured *in vitro* in cell-free fractions of liver from rats injected with thyroxine.

2. In the fraction from which particles sedimenting at 10,000 *g* had been removed, there was an increase in cholesterol synthesis from acetate after 20 μg of thyroxine and a fall after 30 and 50 μg. Fatty acid synthesis was depressed after 20, 30 and 50 μg of thyroxine.

3. The depression of synthesis of cholesterol and fatty acids was due to an effect in the particles sedimenting at 104,000 *g* and could be partially reversed by addition of glycogen; removal of glycogen from the cell-free preparations led to a depression of synthesis.

4. It is suggested that the depression of synthesis brought about by treatment with thyroxine is due mainly to a shortage of glycogen in the liver cells.

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OXYGEN CONSUMPTION IN NEW-BORN RATS

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The rectal temperature of the new-born rat closely approximates to that of its environment. The ability of the rat to maintain a relatively constant body temperature in the cold is fairly well developed by the age of 3 weeks. Adolph (1957) concluded, largely from the work of Antoschkina (1939) and Fairfield (1948), that the new-born rat does not respond to cold with an increase in heat production (as indicated by an increase in O_2 consumption) until the age of 3–4 days. If this were true, then the new-born rat would differ from the new-born of most mammalian species in this respect. Rabbits (Giaja, 1925; Adamsons, 1959), cats (Leichtentritt, 1919; Hill, 1959), dogs (Gelineo, 1957; McIntyre & Ederstrom, 1958; Adamsons, 1959), pigs (Mount, 1959), sheep (Cross, Dawes & Mott, 1959), rhesus monkeys (Dawes, Jacobson, Mott & Shelley, 1960) and human infants (Day, 1943; Brück, Brück & Lemtis, 1958) all show a metabolic response to cold shortly after birth. Adolph's conclusion does not agree with the findings of Gelineo & Gelineo (1951) and Hahn & Koldovský (1958) which suggest that the new-born rat is to some extent capable of increasing O_2 consumption in the cold shortly after birth.

As the new-born rat is now so widely used in experimental work it was decided to determine in more detail the influence of age on minimal O_2 consumption, on the neutral environmental temperature at which O_2 consumption is minimal, and on the maximal increase in O_2 consumption that is observed with cooling. Observations were also made on the effect of varying the O_2 content of the inspired air on the O_2 consumption of young rats at the neutral environmental temperature, and on the effect of breathing 50% O_2 on the metabolic response to cold. A preliminary account of this work has been given (Taylor, 1960).

METHODS

Wistar rats of either sex were placed in a glass chamber which formed part of a closed system of about 300 ml. volume, through which air was circulated at approximately 1·5 l./min by means of an aquarium aerator. Carbon dioxide was absorbed by soda-lime, and the oxygen consumed in the system was replaced automatically. As O_2 was consumed

the movement of a sensitive spirometer attached to the system opened a mercury contact, which started a roller-pump; this introduced O_2 into the system until the mercury contact was closed once more. The roller-pump was fed with oxygen from a second spirometer which was refilled once every 4 min. The movements of both spirometers were recorded on a smoked drum, as illustrated in Fig. 1. The closed system was almost entirely immersed in a water-bath maintained at constant temperature to within 0·1° C. After abrupt changes of bath temperature of 2° C the temperature within the system reached a new equilibrium within a few minutes. The O_2 content of the system was changed by flushing with O_2:N_2 mixtures at 1·5 l./min for 2–3 min, and the O_2 content of the effluent gas was measured with a Beckmann O_2 analyser, which was checked by gas analysis with a Haldane apparatus. The rate of O_2 consumption was corrected to and is expressed as dry gas at s.t.p.

Rats were removed from the nest immediately before the start of an experiment. The mother always accepted them back if they were returned at the end of the experiment. Usually 6–7 new-born rats were placed in the respiration chamber at one time; fewer rats were put in the chamber as their age and size increased. Three-week-old rats were, with one exception, investigated singly. Thus the total weight of the rats, or rat, used for each experiment was usually 30–40 g. The environmental temperature was lowered progressively in steps of 1–5° C (Fig. 2). O_2 consumption was recorded for three or more 4-min observation periods after a steady rate of O_2 consumption was reached following each change in environmental temperature. In experiments on the influence of the O_2 content of the inspired air on O_2 consumption, each test period was bracketed by periods during which the rats breathed room air. Blood was obtained by decapitation. Lactate was measured by Barker & Summerson's (1941) method, and glucose by the glucose–oxidase method (Huggett & Nixon, 1957).

RESULTS

Breathing air

All rats, even on the first day of life, showed an increase in O_2 consumption on exposure to a cold environment (Fig. 1). Figure 2 shows the rate of O_2 consumption in three experiments on rats aged 4 hr, 23 hr and 21 days (at the beginning of the experiment), at different environmental temperatures. The 4-hr-old rats (×) showed only a very small increase in O_2 consumption between 38 and 31° C. In the 23-hr-old rats (○) there was a large increase in O_2 consumption when the temperature was reduced below 36° C, and

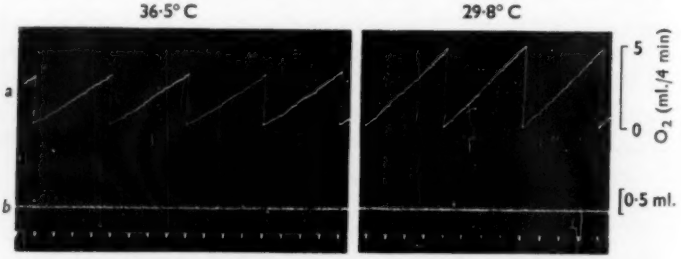

Fig. 1. Six rats, 19 hr old, 32·7 g in all. Records of O_2 consumption (*a*) and of closed-circuit volume (*b*) at an environmental temperature of 36·5° C (within the neutral zone) and at 29·8° C. Time marker, 1 min.

there appeared to be a neutral zone between 36 and 38° C. In the 21-day-old rat (●) the neutral zone extended from about 34 to 38° C, and below this there was a progressive increase in O_2 consumption down to 11° C, the lowest temperature studied.

These experiments illustrate the change in metabolic response to a lowered environmental temperature with increasing age. In the youngest rats, less than 6 hr old, which had not been suckled, there was only a trivial

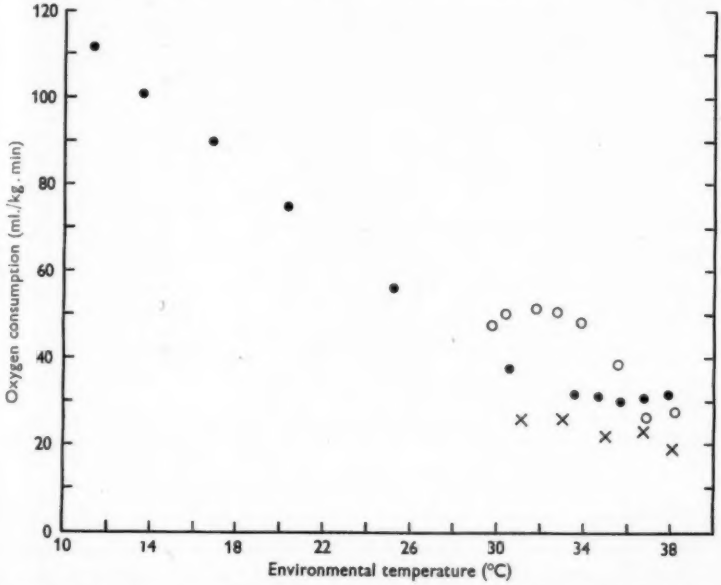

Fig. 2. Measurement of O_2 consumption per unit body weight at different environmental temperatures in a group of rats 4 hr old (×), in rats 23 hr old (○) and one rat 21 days old (●).

increase in O_2 consumption, and it was often difficult to define the neutral zone. Only five experiments on this point are illustrated in Fig. 3, in which are shown the minimal O_2 consumption at the neutral temperature (△) and the maximal O_2 consumption observed in the cold (▲). However, a number of other observations at this age confirmed these findings.

Between 6 and 48 hr of age there was a small increase in the minimal rate of O_2 consumption recorded at the neutral temperature, from a mean of 19·7 to 28·7 ml./kg.min (Fig. 3, △, □, ○). There was little further change over the next 3 weeks. The maximal oxygen consumption on exposure to cold increased rapidly in rats which had been suckled (i.e. more than 6 hr old) to about 50 ml./kg.min at 48 hr of age, and thereafter more slowly

(Fig. 3, ●, ■). In rats which were 3 weeks old O_2 consumption increased from about 30 ml./kg.min at the neutral temperature to as much as 112 ml./kg.min in the cold. This may not have been the maximum possible O_2 consumption because, as in the experiment illustrated in Fig. 2 (●), the environmental temperature was not reduced below 10° C.

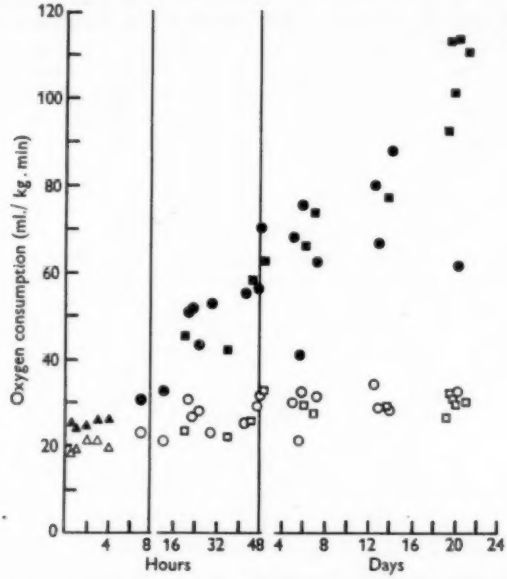

Fig. 3. The minimal (△, ○, □) rate of O_2 consumption at the neutral temperature and the maximal rate (▲, ●, ■) in a cool environment has been plotted for rats of different ages, before suckling (△, ▲) and placed in the respiration chamber either together (○, ●) or separated by partitions (□, ■).

Figure 4 shows the range of the neutral temperature zone (vertical bars) and the temperature at which O_2 consumption was maximal (×) at different ages. The neutral zone varied considerably in rats of the same age. Five of the seven litters of ½–12-hr-old rats had relatively low neutral environmental temperature zones (33–35·5° C) while the other two litters of rats of this age had higher neutral environmental temperatures, similar to those observed for rats 18 hr to 14 days of age. Three-week-old rats had lower neutral environmental temperature zones, ranging from 30·5 to 36° C.

The environmental temperature at which the maximal rise in O_2 consumption was observed increased from 29·6 ± 0·7° C (S.E.) in ½–12-hr-old rats to 31·1 ± 0·4° C in rats 18–51 hr old. This difference is not significant.

There was a fall in this temperature between 2 and 5 days after birth which continued to 3 weeks. The figure given for the temperature at which O_2 consumption was maximal in four of the six 3-week-old rats may be too high.

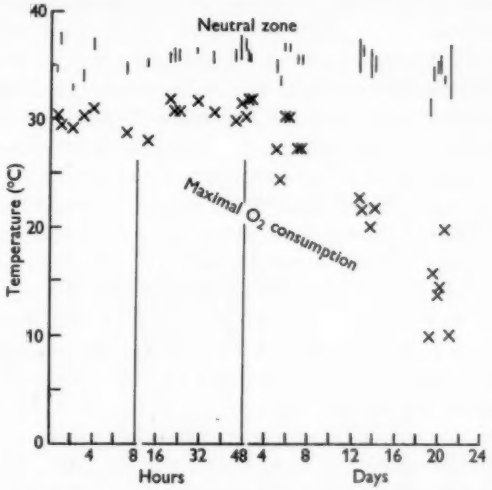

Fig. 4. The range of neutral environmental temperatures (vertical lines) and the temperature at which O_2 consumption was maximal (×) plotted against age from birth.

Hill & Hill (1913) showed that, when 2-month-old rats were placed together in a calorimeter, their heat production dropped considerably below that of rats studied singly. Litters of rats 1–14 days old were therefore divided, and one half was placed together in the respiration chamber while the other half was segregated, one from the other, by double partitions. In a number of such experiments the rates of O_2 consumption were compared at the neutral temperature and in the cold. The differences observed were such as to be expected, in that the neutral temperature was a little lower and the maximal increase in O_2 consumption was a little higher in the grouped than in the separated litter-mates. However, as Fig. 3 (□, ■) shows, these differences were small, particularly when compared with the very large changes observed with increasing age.

The rats were in direct contact with the glass wall of the respiration chamber, which was immersed in water at a temperature about 0·5° C lower than that of the air in the respiration chamber. Antoschkina's (1939) observations suggest that the rectal temperature of the rats would be 1–2° C higher. It seemed possible that the rats might lose heat rapidly

because they were normally placed in direct contact with the glass, which has a relatively high thermal conductivity. To test this possibility groups of young rats were placed alternately in the glass cylinder or in a Perspex cylinder lined with cardboard, both at the neutral temperature and in the cold. There was no significant difference in O_2 consumption in the two cylinders.

The rats were least active at the neutral temperature; the unfed new-born rats were more active than any others. As the temperature was reduced, by steps of 1–2° C, all the rats showed increased activity, which usually subsided somewhat within a few minutes. With extreme reduction of temperature, to below 30° C in new-born rats and below 15° C in 3-week-old rats, they became torpid. The rate of respiration, and the depth so far as it could be judged, increased in all rats in a cool environment. Slight shivering was always seen in 3-week-old rats below 20° C, but not in younger rats. Rats less than 12 days old have little hair; pilo-erection was apparent in the cold by 2 weeks of age, when blanching of the skin was also seen. In new-born rats the skin was very red and did not change colour when the environmental temperature was reduced to just below 30° C, when O_2 consumption began to decrease.

When the environmental temperature was raised above the neutral zone, as shown by an increase in O_2 consumption, there was a considerable increase in activity at all ages. This increased activity persisted in very young rats, but soon subsided in older ones. The respiration rate increased in rats of all ages.

Breathing O_2 at high or low concentrations at the neutral temperature

Rats 0–1, 3–4 and 14–15 days old were exposed at the neutral temperature, which was determined while breathing room air in each experiment, to 50, 18, 15 and 10 % O_2 in that order. Each period of exposure was preceded and followed by 12–20 min periods, during which the rats breathed room air, as is shown in Fig. 5. The O_2 consumption while breathing room air remained constant during these experiments within 1–2 ml./kg. min, except after exposure to 10 % O_2.

Inhalation of 50 % O_2 was usually associated with a slight increase in O_2 consumption (Fig. 6). There did not appear to be any systematic difference with age. Most of the rats increased their activity on exposure to 50 % O_2; the 0–1-day-old rats sustained this increase for the period of exposure, but the activity of the older rats soon returned to the level observed when they were breathing room air.

A reduction in the O_2 concentration of the inspired air to 18 % or less in 0–1-day-old rats, in all but one litter, caused a considerable fall in O_2 con-

sumption (Fig. 6). There was an even greater fall when the O_2 concentration was lowered further. In older rats O_2 consumption was well maintained on 18 and 15% O_2, but there was still a profound fall on 10% O_2. All the rats showed increased activity and cyanosis on 15% O_2; some of them became ashen coloured and developed slow gasping respiration on 10% O_2. Even a short period of exposure to 10% O_2 at the neutral temperature caused some deaths.

In three litters each of 8–10 rats 20–45 hr old the neutral temperature was determined while breathing room air. Half of each litter was removed

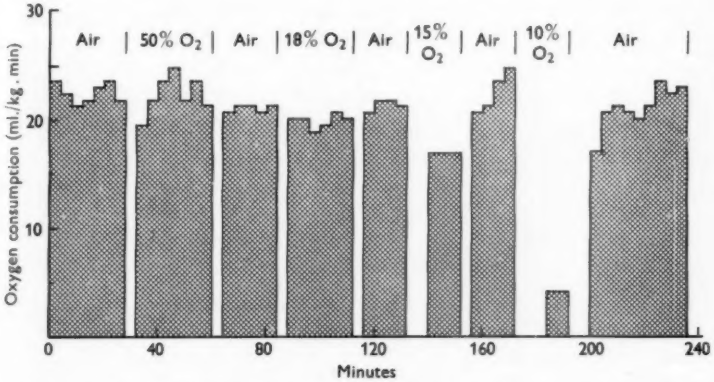

Fig. 5. Nine rats, 1 hr old, 48·0 g in all, at their neutral temperature, 35·7° C. Administration of 50% O_2 caused little change in O_2 consumption, but 18% O_2 or less caused a fall in O_2 consumption.

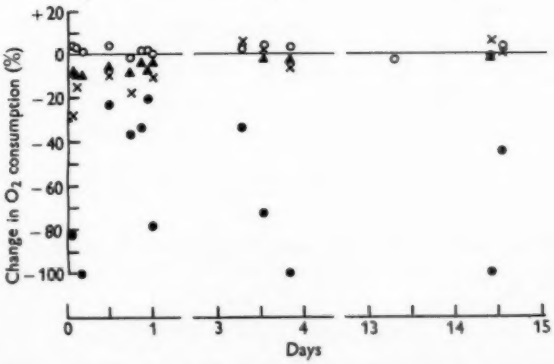

Fig. 6. Observations on rats of different ages, all at the neutral temperature, of the change (%) in O_2 consumption as compared with that breathing room air, on exposure to 50% O_2 (○), 18% O_2 (▲), 15% O_2 (×) or 10% O_2 (●).

and the blood lactate was determined. The other half was exposed to 15% O_2 for ½ hr, and the blood lactate was then found to be considerably higher than that of their litter-mates (Table 1). In only one rat breathing air was the blood lactate as high as that of any of its litter-mates breathing 15% O_2. There was no significant difference between the blood glucose levels of the two groups of rats.

TABLE 1. Mean blood lactate levels in new-born rats exposed to air or to 15% O_2 for ½ hr

Litter	Mean blood lactates (mg/100 ml.) Breathing air	Breathing 15% O_2
A	14·1	23·4
B	13·7	19·2
C	14·9	31·5

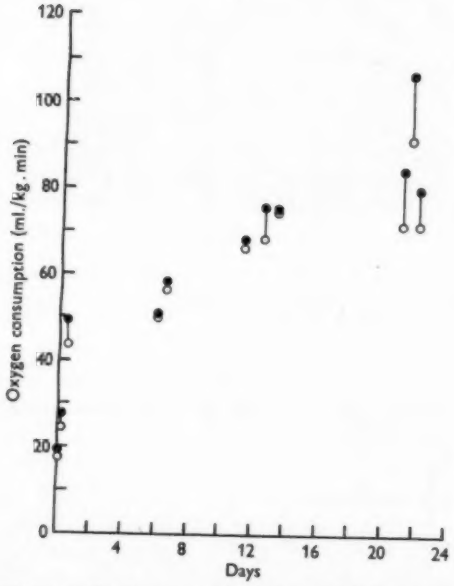

Fig. 7. Observations on the O_2 consumption of rats of different ages, at temperatures such that O_2 consumption was near maximal, breathing either room air (○) or 50% O_2 (●).

Breathing 50% O_2 in the cold

Rats 0–½, 6–7, 11–13 and 21–22 days old were exposed for about ½ hr to 50% O_2, at the environmental temperatures predicted (from Fig. 4) to stimulate the maximal metabolic response to cold. Each period of ex-

posure to 50 % O_2 was bracketed by periods of about ½ hr during which the rats breathed room air.

Breathing 50 % O_2 (Fig. 7, ●) was associated in each experiment with a small increase in O_2 consumption, when compared with the mean of the O_2 consumption values for the periods when the rats breathed room air (Fig. 7, ○). The absolute increase in O_2 consumption was greatest in the oldest rats. However, when expressed as a proportion of the control values, both 0–½ and 21–22 day-old-rats had increases of 9–15 %, and most of the 6–7 and 11–13-day-old rats had smaller increases. All the rats showed increased activity on 50 % O_2. The 21–22-day-old rats, which shivered slightly in room air, began to shiver more vigorously and more frequently when placed in 50 % O_2; shivering became weaker when they were returned to room air. The 11, 12 and 13-day-old rats did not shiver when breathing room air, but began to shiver slightly several minutes after they were put in 50 % O_2; shivering then persisted when they were returned to room air. The 0–½ and 6–7-day-old rats did not shiver on exposure to the cold in either air or 50 % O_2.

DISCUSSION

The metabolic response to cold

The results demonstrate that the O_2 consumption of the new-born rat increases when the environmental temperature is lowered from 35 to 29° C, thus confirming the findings of Gelineo & Gelineo (1951). Antoschkina (1939) likewise found an increase in the O_2 consumption of 2–7-day-old rats when the environmental temperature was dropped from 35 to 20–30° C, and Barić (1953) observed an increase in the O_2 consumption of 4–5-day-old rats when the ambient temperature fell from 38 to 30° C. On the other hand, both Gulick (1926) and Fairfield (1948) concluded that the rat does not increase O_2 consumption on exposure to cold for the first few days of life. It is likely that the large and abrupt drop in the environmental temperature from 35 to 20° C in Fairfield's experiments obscured, by rapid tissue cooling, any transient increase in O_2 consumption that her 0–3-day-old rats might have shown.

There was an increase in the metabolic response of the rat to cold between the ages of 4 and 24 hr (Fig. 3). This also is consistent with the observation by Gelineo & Gelineo (1951) that rats 3–5, 13–15, and 23–25 hr old, at an environmental temperature of 29–30° C, had O_2 consumptions of 26, 42 and 57 ml./kg.min, respectively. The further, more gradual, increase in the rat's maximal O_2 consumption in the cold between 24 hr and 3 weeks of age agrees well with Antoschkina's findings (1939). Recalculation of her data shows that the maximal O_2 consumption in the cold rose from 62 ml./kg.min in 2–7-day-old rats to 112 ml./kg.min in 3–4-week-old

rats. The metabolic response to cold also increases in dogs from birth to 3 weeks of age (Gelineo, 1957; McIntyre & Ederstrom, 1958), and also in monkeys (Dawes *et al.* 1960), and in human infants from birth to 9 days of age (Brück *et al.* 1958).

The most striking increase in the metabolic response of new-born rats to cold occurred soon after they had begun to feed. Barić (1953) found that 4–5-day-old rats, unfed for 24 hr, showed a decrease in O_2 consumption when the environmental temperature was lowered from 38 to 30° C. Mourek (1958) also observed a large decrease in O_2 consumption in 2–3-week-old rats studied at 26° C after a 24-hr fast. Therefore the small metabolic response to cold in unfed rats may be related to lack of carbohydrate reserves. Certainly there is a very large rapid fall in liver glycogen in rats after birth (Stafford & Weatherall, 1960).

Factors other than feeding might modify the metabolic response to cold during the first hours of life. The new-born rabbit (Adamsons, 1959) and the new-born lamb (Cross *et al.* 1959) show a considerable increase in both the minute volume of breathing and in O_2 consumption when changed from the neutral to a cold environment. An inadequate ventilatory response might limit the O_2 consumption of the new-born rat, as might the pulmonary diffusion capacity for O_2, the rate of pulmonary capillary blood flow, or the transfer of O_2 from the blood to the tissues. Exposure of new-born rats in the cold to 50% O_2 caused only a small increase in O_2 consumption, less than the increase which was observed with increasing age (Fig. 7). It therefore seems unlikely that the metabolic response to cold in new-born rats is limited to any significant extent by the uptake of O_2 by the lungs. It is probable that the increase in the metabolic response to cold with age represents, in part, an adaptation to the change in environmental temperature from about 38° C *in utero* to the nest temperature of about 32° C.

The gradual increase in the maximal O_2 consumption in the cold between 1 day and 3 weeks of age takes place during a time of rapid improvement in the rat's ability to maintain a relatively stable body temperature in the cold. This improvement in thermoregulation is due, in part at least, to factors that reduce heat loss (Adolph, 1957). Therefore it is not possible to conclude that the maximal potential for heat production of a 3-week-old rat is superior to that of a 1-day-old rat at a given cool environmental temperature, since the heat production of the younger rat will be depressed to a greater extent than that of the older rat by its lower body temperature. Nor is it justifiable to compare the O_2 consumption of immature rats of different ages at the same body temperature, as the environmental temperatures, and therefore the skin temperature gradients (which probably provide stimulation for increased heat production), will be different at different ages.

The results also confirm Antoschkina's observations (1939) on the progressive decrease in the environmental temperature at which the maximum metabolic response to cold occurs between birth and 3 weeks of age. The progressive improvement in thermoregulation in the cold explains this change.

Minimal O_2 consumption in new-born rats

Three studies carried out on young rats at or near the neutral temperature must be considered. Gelineo & Gelineo (1951) found an increase in O_2 consumption from 19 to 28 ml./kg.min between the ages of 3–5 and 23–25 hr in rats investigated at 35–36° C. Fairfield (1948) in experiments at 35° C observed an O_2 consumption of from 21 to 36 ml./kg.min in rats 0–17 days of age, and found no correlation between O_2 consumption and age. Antoschkina (1939) noted mean values of 36–42 ml./kg.min in 2-day–3-week-old rats at 35° C. The minimal O_2 consumption increased 32% in the present investigation, from a mean of 19·7 ml./kg.min in 0–7-hr-old rats to a mean of 28·7 ml./kg.min in 2-day-old rats; this agrees well with the findings of Gelineo & Gelineo. The data on minimal O_2 consumption for rats 18 hr–3 weeks old are within the range of Fairfield's observations and below the mean values given by Antoschkina. The explanation for this discrepancy is probably that some rats from 1 to 14 days of age have their neutral temperature above 35° C, at which Antoschkina made her measurements.

An increase in O_2 consumption shortly after birth has been found in all the mammalian species in which this has been investigated. This increase is two- to threefold in lambs (Dawes & Mott, 1959), monkeys (Dawes *et al.* 1960) and pups (Gelineo, 1957) studied at the neutral temperature. Mount (1959) noted a 50% increase in O_2 consumption in pigs at 30° C (several degrees below their neutral environmental temperature) between 10–18 hr and 1–6 days. It can be estimated from his Fig. 3 that a 20% increase in O_2 consumption occurred over this period of time in pigs studied at 36–38° C, which is at or above their neutral temperature. Brück *et al.* (1958) found a trivial increase in the O_2 consumption of full-term human infants between birth and 4–6 days of age. They were investigated at 32–35° C, which may be the neutral environmental zone for the infant, though more information is required. Cross, Tizard & Trythall (1958) noted a 20% increase in O_2 consumption between birth and 3 days of age in full-term and premature infants, but it is likely that these infants were below the neutral environmental temperature.

It will be seen that the increase in minimal O_2 consumption after birth is certainly much less in rats than it is in lambs, monkeys and puppies. This might be due to the relative immaturity of the rat at birth, but it is

also pertinent that the rat increases in weight about sixfold during the first 3 weeks of life. Consequently we should expect the ratio of surface area to weight to be halved during this period and, other things being equal, the metabolic rate per unit body weight to decrease in proportion.

The neutral temperature zone in young rats

One of the principal reasons for undertaking the experiments which have been described was to define the neutral temperature zone in rats, its variation with age and its variation from one litter to another at the same age. The neutral environmental temperature drops from about 35° C at 2 weeks of age (Fig. 4) to the adult range of 28–30° C between 6 weeks and 2 months of age (Benedict & MacLeod, 1928; Antoschkina, 1939). The beginning of the fall in neutral environmental temperature coincides in time with development of effective insulation by the young rat (Adolph, 1957). The neutral temperature is also higher in the new-born dog (McIntyre & Ederstrom, 1958) and in the new-born human baby (Brück *et al.* 1958) than in the adult of these species.

The difference that may occur between neutral temperature zones in young rats of the same age (Fig. 4) makes it imperative that the neutral zone should be defined in each experiment if one wishes to speak of minimal O_2 consumption with certainty. In some instances the neutral zone for rats during the first day of life was above 36° C; it was about 34° C at 3 weeks of age. The investigations carried out on young rats at environmental temperatures of 31° C or less, with the intention of determining the influence of age on minimal metabolism, must be considered in this light (Davis, 1937; Kibler & Brody, 1942; McCashland, 1951; Grad, 1953; Klieber, Smith & Chernikoff, 1956).

For the same reason, the observations of Mourek (1959) are also difficult to interpret. He measured the respiratory rate in rats of different ages, breathing air and during hypoxia, at 32 and 26° C. He chose 32° C as the most usual nest temperature (which would agree with my observations). However, 32° C was below the neutral environmental temperature for almost all rats aged 0–21 days (Fig. 4), and Dawes & Mott (1959) and Adamsons (1959) have shown that the ventilatory response of young rabbits to hypoxia varies with the environmental temperature below the neutral zone.

It is well known that the new-born rat will survive total anoxia in nitrogen for a very long period, about 28 min at 35° C (Stafford & Weatherall, 1960). The survival time, to the last gasp, falls rapidly after birth to reach the adult value of 2–3 min at just under 3 weeks. One of the possible factors in this remarkable change is the increase in minimal metabolic rate

after birth. The present experiments suggest that this is unlikely to be a factor of much importance, since in the rat the increase in minimal O_2 consumption is small, and occurs within 2 days of birth.

O_2 consumption and hypoxia at the neutral temperature

The decrease in O_2 consumption of the new-born rat at the neutral environmental temperature when placed in 18 and 15 % O_2 was unexpected. The minimal O_2 consumption of young kittens (Hill, 1959), and of new-born rabbits (Adamsons, 1959), lambs (Cross *et al.* 1959) and monkeys (Dawes *et al.* 1960) is not reduced until the O_2 concentration of the inspired air is lowered to about 10 % at the neutral temperature. New-born human infants show a decrease in heat production (Brodie, Cross & Lomer, 1957) and a decrease in O_2 consumption (Cross *et al.* 1958) when breathing 15 % O_2. It is not known that these infants were at the neutral environmental temperature; the extra metabolism in the cold is very susceptible to hypoxia.

Mourek (1959) has investigated the effect of breathing 10 % O_2 on the O_2 consumption of young and adult rats at 32–33° C, i.e. well below the neutral environmental temperature for the youngest rats, and above the neutral temperature for adult rats. He found a 70 % decrease in O_2 consumption in rats at birth, a progressively smaller decrease up to 4 weeks of age, and no decrease in adult rats. His results may be, in part, a reflexion of the decrease in the rat's neutral environmental temperature that begins at about 2 weeks of age. It is also difficult to compare the present values with the results of others on the effect of hypoxia on O_2 consumption of adult rats. Most of these studies were done at environmental temperatures of 22–25° C (Blood, Elliott & D'Amour, 1946; Rothschuh, 1947; von Flückiger, 1956); the neutral temperature zone of the adult rat is 28–30° C (Goto, 1923; Benedict & MacLeod, 1928). Lintzel (1931) has shown that the O_2 consumption of adult rats at low atmospheric pressure is much reduced at environmental temperatures below the neutral temperature. It can at best be inferred from these papers that a moderate decrease in O_2 consumption can be expected in adult rats when the O_2 concentration of the inspired air is lowered to about 10 % at or slightly below the neutral temperature.

O_2 consumption was well maintained in 3·5- and 14-day-old rats breathing 18 and 15 % O_2 at the neutral temperature. The most likely explanation of this difference between the new-born and the 3·5-day-old rat is that the ventilatory response to hypoxia increases over this time. Adolph (1957) has reported a marked increase in pulmonary ventilation in 1–2-day-old rats exposed to a low O_2 pressure (44 mm Hg $\simeq$ 6 % O_2), which regresses to near control values in a few minutes. Six- to nine-day-old rats respond

better to the same O_2 pressure and maintain the increased ventilation for an hour or more. The depth of breathing increased while respiratory rate changed little in these experiments. Yet these observations were made at an environmental temperature of 33° C or less (Adolph & Hoy, 1960) and hence may not be applicable to the present observations.

The decrease in O_2 consumption of new-born rats breathing low-O_2 mixtures could signify either inadequate uptake of O_2 (in which case blood and tissue pO_2 would fall and the resulting energy debt might be partially met by anaerobic glycolysis) or a decrease in blood flow to certain regions of the body. New-born rats are certainly hypoxaemic when breathing 15 % O_2, as they show generalized cyanosis and an increase in blood lactate (Table 1). This does not exclude the possibility that reduction of flow in certain vascular beds might further reduce the O_2 consumption of young rats in 15 % O_2.

O_2 consumption, shivering and hyperoxia in the cold

Gulick (1926) states that rats first shiver in the cold shortly before 11 days of age. In the present experiments shivering was noted regularly in 19–22-day-old rats, but was not seen in 12–14-day-old rats breathing room air. Observations on the increase in, or initiation of, shivering in young rats breathing 50 % O_2 are in accord with von Euler & Söderberg's (1958) finding that anaesthetized adult cats shiver more while breathing 100 % O_2. The modest increase in O_2 consumption of young rats breathing 50 % O_2 may be explained by the initiation or augmentation of shivering in 11–22-day-old rats, and the increase in activity seen in 0–22-day-old rats.

Mourek (1959) has compared the O_2 consumption of young and adult rats when breathing air or 100 % O_2 at environmental temperatures of 32–33° C. Rats 0–3 days old had a 22 % increase in O_2 consumption, while rats 3–4 weeks old and adult rats showed no change in O_2 consumption on 100 % O_2. The most likely explanation for the observations is the decrease in the neutral environmental temperature of rats that takes place between birth and 3 weeks of age (Fig. 4). Rats show little change in O_2 consumption on 50 % O_2 at the neutral temperature (Fig. 6) and Mourek's 3–4-week-old rats must have been at or near the neutral temperature.

SUMMARY

1. The rate of O_2 consumption was measured in unanaesthetized rats from birth to 22 days of age, at the neutral temperature and in the cold.

2. The mean minimal O_2 consumption, at the neutral temperature, of new-born rats which had not been suckled was 19·7 ml./kg.min. It rose to 28·7 ml./kg.min by 2 days, and remained at this level up to 3 weeks from birth.

3. The neutral temperature zone extended over a range of up to 2° C between 33 and 38° C for the first 6 days of life. It covered a wider and lower temperature range by 3 weeks of age.

4. On exposure to cold there was always a rise in O_2 consumption. This rise was small in rats which had not been suckled; it increased rapidly from 6 hr of age onwards. The rise was even greater by 21 days of age while the environmental temperature at which O_2 consumption was maximal fell from about 30° C to below 20° C.

5. At the neutral temperature exposure of 0–1-day-old rats to 18%, 15 or 10% O_2 in place of room air, caused a fall in O_2 consumption. Rats 3–14 days old maintained their O_2 consumption well on 18 or 15% O_2, but there was a profound fall on 10% O_2. The O_2 consumption of young rats did not change significantly on 50% O_2.

6. In a cold environment, at which O_2 consumption was near maximal, exposure of 0–22-day-old rats to 50% O_2 caused an increase in O_2 consumption.

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ESTIMATION OF TOTAL BODY WATER (VIRTUAL TRITIUM SPACE) IN THE RAT, CAT, RABBIT, GUINEA-PIG AND MAN, AND OF THE BIOLOGICAL HALF-LIFE OF TRITIUM IN MAN

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Isotopes of water (deuterium oxide and tritium oxide) have been used for the determination of total body water in certain mammalian species (Hevesy & Hofer, 1934; Pace, Kline, Schackman & Harfenist, 1947; Soberman, Brodie, Levy, Axelrod, Hollander & Steele, 1949; Haigh & Schnieden, 1956; Langham, Eversole, Hayes & Trujillo, 1956; Fallot, Aeberhardt & Masson, 1957). However, the techniques involved in these determinations have limited use, being either difficult, expensive or time-consuming. Recent advances in scintillation counting techniques (Haigh, 1957) have made it much easier to estimate one of these isotopes of hydrogen, namely tritium. The purpose of the present paper is to describe how far such scintillation counter techniques can be applied to the determination of tritium in urine and plasma and the estimation of degree of error involved. The use of such techniques for a comparative study of total body water in a number of mammalian species and of water turnover under tropical conditions in one species, namely man, is also presented.

METHODS

0·5 ml. of tritiated water or processed tritiated plasma or urine (see below) was added to 8 ml. of absolute ethanol and 10 ml. of scintillation solution (3 g 2:5-diphenyloxazole/l. sulphur-free toluene). The mixture was shaken and then centrifuged at approximately 1500 rev/min for 2–3 min. 5 ml. of the supernatant fluid was placed in the special counting container supplied with the N612 Ekco Scintillation Counter. This scintillation unit was kept at −20° C in a deep-freeze cabinet and its amplifier set at maximum gain. It was connected to a N530E Ekco Automatic Scaler unit which was set at H.V. 1200 and Discrimator bias of −5 V. The scaler received a stabilized current from a constant-voltage stabilizer (Servomex AC 2 Mark IIB). Unless otherwise stated, all specimens were counted for 1000 sec and were allowed to cool in their counting containers for 1000 sec at −20° C before counting.

Preparation of urine. 3·5 ml. of urine was mixed thoroughly in a test-tube with 0·5 g of activated charcoal. The resultant sludge was filtered through a Whatman No. 2 filter paper and 0·5 ml. of the filtrate was then added to 18 ml. of the alcohol-scintillator mixture, shaken and centrifuged, and a 5 ml. portion taken for counting.

Preparation of rat, cat, guinea-pig or rabbit plasma. 0·5 ml. of plasma was added to 18 ml. of the alcohol-scintillator solution mixture. After thorough shaking the mixture was centrifuged. 5 ml. of the clear supernatant fluid was then withdrawn and placed in the counting container before counting.

Preparation of human plasma. Since it was found that the above method of preparation for rat plasma gave a coloured (orange-yellow) supernatant liquid with human and baboon plasma, another method was also investigated. The plasma was precipitated with 10% trichloroacetic acid (Langham *et al.* 1956) and 0·5 ml. of the clear colourless filtrate was mixed with 18 ml. of alcohol-scintillation fluid mixture. 5 ml. of this final mixture was used for counting.

Recovery experiments. In order to determine the degree of quenching resulting from these procedures for plasma and urine, known amounts of tritiated water were added to urine, plasma or distilled water and the activities of these solutions (after treatment, if necessary) compared. The final tritium concentration ranged from 50 to 150 μc/ml. In no instance was the urine or plasma diluted by the tritiated water by more than 10%.

Determination of virtual tritium space

Rat. Male or female albino rats weighing between 150 and 250 g were used. These were given 10 μc of tritiated sodium chloride solution (0·9 g sodium chloride in 100 ml. tritiated water) either intraperitoneally, or intravenously. The intravenous injection was given through a polythene cannula inserted into the jugular vein 18 hr previously. Following the administration of the tritiated water the animals were left for 2 hr and then given 5 ml. of water orally. All urine passed up to 2½ hr after injection of the tritiated sodium chloride solution was rejected, but the first 3·5 ml. of urine passed after 2½ hr was collected. (Preliminary experiments had shown that after 2½ hr tritiated water given by the above routes had reached equilibrium in the rat.) The rat was then weighed and killed, and the urine processed as stated above. After counting, the virtual tritium space at approximately 2½ hr was calculated.

As it was desirable to compare this space with a direct determination of total body water, the corpse, after mincing, was placed in an oven kept at $100 \pm 2°$ C and dried to constant weight; care being taken to trap the tritiated water vapour.

Cat. Young adult cats of both sexes were used (wt. 1·0–2·25 kg). They were starved during the night and the following morning tritiated sodium chloride solution (50 μc/kg) was administered intraperitoneally in a volume of not more than 2 ml. After 2½ hr the animals were anaesthetized with pentobarbitone sodium and a sample of blood was withdrawn. From two of the cats a further blood sample was withdrawn at 3 hr, to check whether any marked change had occurred between 2½ and 3 hr in the tritium concentration of the plasma. Plasma solids were determined by drying six samples of plasma at 100° C to constant weight.

Guinea-pig. Young adults were used of weight ranging between 300 and 420 g and of both sexes. An intraperitoneal dose of tritiated sodium chloride solution (50 μc/kg) was given and a procedure was used similar to that employed for the determination of cat body water.

Rabbits of both sexes (wt. 1·6–2·3 kg) were used. An intraperitoneal dose of tritiated sodium chloride solution (50 μc/kg) was given and a procedure similar to that for the determination of cat body water was used except that the animals were not anaesthetized and blood samples were taken from the ear.

Human beings. Healthy male students, aged between 21 and 24 yr and weighing between 47 and 64 kg, each drank a glass of water (approx. 500 ml.) containing 3 mc of tritiated water 1 hr after breakfast. Urine samples were collected just before the experiment started and at quarter- or half-hourly intervals for up to 5 hr afterwards. Recovery determinations were performed on the samples of urine taken before the experiment started and the figures obtained were used to correct for tritium content in the urine subsequently collected. (This assumed that the quenching factor remained constant throughout the experiment.)

RESULTS

Figure 1 shows that a straight-line relationship exists between tritium concentration in urine and recorded count up to a concentration of tritium in urine of 500 nc/ml. Similar experiments show a straight-line relationship for tritium in water over the same range and also for tritium in plasma up to 100 nc/ml. (this being the maximum concentration of tritium in plasma investigated). Each sample was counted for 1000 sec. It was found that an individual count at a concentration of 40 nc/ml. was subject to an experimental error of $\pm 2\%$ (S.E. of the mean of 8 counts). This was the lowest tritium concentration met with in practice. The S.E. was slightly less at higher tritium concentrations.

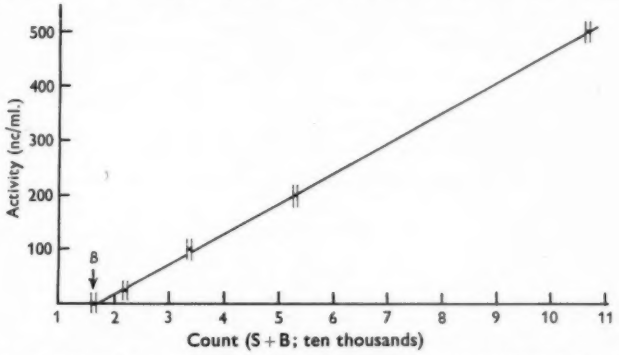

Fig. 1. Relationship between concentration of tritium in urine and the mean count observed for 1000 sec. Horizontal distance between parallel lines represents S.E. of 10 such counts. In all figures S equals true count of specimen, B equals background count.

Table 1 shows the percentage recovery of tritium from various body fluids in several species. In any of the species investigated a higher percentage recovery is observed from plasma than from the urine.

Table 2 compares the virtual tritium space of the rat with direct measurement of total body water obtained by a desiccation procedure. It can be seen that the virtual tritium space estimated after both intravenous and intraperitoneal injection of tritium is approximately 4% higher than the total body water obtained by desiccation.

Table 3 compares the values for virtual tritium space (total body water) in a number of mammalian species.

Figure 2 shows the concentration of tritium in the urine with time (up to 5 hr) in three different subjects following the oral administration of tritiated water. In all ten human beings equilibrium was obtained by the third hour after administration.

TABLE 1. Recovery of tritium from urine and plasma (taking tritium in water as 100%; mean ± S.E.)

Fluid	Recovery (%)
Water	100 (10)*
Rat urine	95·6 ± 0·4 (10)
Rat plasma	99·4 ± 0·5 (10)
Cat plasma	100 ± 0·6 (10)
Rabbit plasma	97·6 ± 1·5 (9)
Human plasma	97·6 ± 1·0 (11)
Human urine	95·2 ± 0·8 (10)
Guinea-pig plasma	97·0 ± 0·5 (10)

* Figures in parentheses indicate the number of samples used in the determination.

TABLE 2. Comparison of virtual tritium space and direct measurement of total body water by desiccation in the rat (mean ± S.E.)

	Total body water (%)		
Route	Direct	Virtual tritium space	Difference
Intravenous	63·9 ± 1·0 (10)*	68·1 ± 1·2 (10)	4·2
Intraperitoneal	66·5 ± 0·8 (7)	70·8 ± 1·3 (2)	4·3

* Figures in parentheses indicate the number of animals used.

TABLE 3. Virtual tritium space (total body water) in mammals (%; mean ± S.E.)

Species	Route	Virtual tritium space
Rat	Intravenous	68·1 ± 1·2 (10)*
Cat	Intraperitoneal	70·6 ± 0·9 (8)
Guinea-pig	Intraperitoneal	75·2 ± 0·9 (18)
Rabbit	Intraperitoneal	65·5 ± 2·3 (7)
Man	Oral	70·8 ± 1·1 (10)

* Figures in parentheses represent number of animals used.

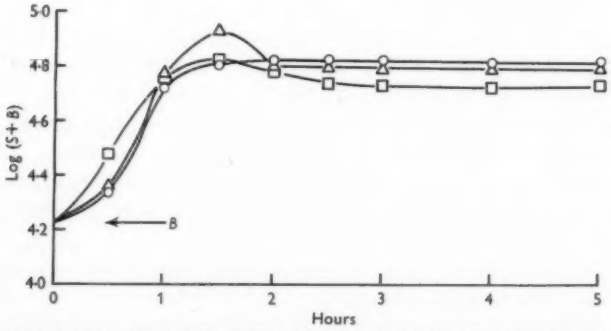

Fig. 2. The relationship between concentration of tritium in the urine and time over a period of 5 hr. Human subjects given a single dose of 3 mc tritiated water at time zero. Specimens were counted for 1000 sec.

Figure 3 shows the daily urinary concentration of tritium over a period of 10 days in two subjects. It can be seen that the biological half-life for tritium for these two subjects was approximately 6 days and 11 days, respectively. The mean value for ten subjects was $7{\cdot}5 \pm 0{\cdot}6$ days (mean $\pm$S.E.), with a range of 5–11 days. The daily maximum temperature recorded during this period ranged from 79 to 93° F (26–34° C) and the minimum temperature from 69 to 75° F (20·6–23·8° C). The relative humidity at 7.00 hr ranged from 96 to 100% and at 16.00 hr from 62 to 83%.

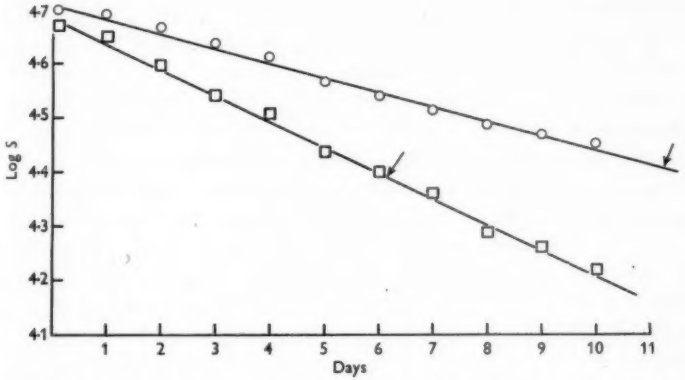

Fig. 3. Daily urinary tritium concentration in two human subjects following a single oral dose of 3 mc tritiated water. Specimens were counted for 1000 sec. Arrows show biological half-life.

DISCUSSION

A number of substances have been used for determining total body water. Theoretically the best estimate would be obtained from a substance which quickly becomes dispersed in the total body water, is capable of easy and accurate estimation in urine or plasma, is not metabolized and behaves physiologically like normal water. It should also have no harmful effects on body tissues.

Whilst sulphonamides, antipyrine and *N*-acetyl-antipyrine are metabolized, deuterium oxide and tritium oxide do not suffer from this disadvantage. They are also relatively non-toxic (Brues, Stroud & Rietz, 1952) and would appear to fulfil most of the requirements of an ideal substance for total body water estimations. Prentice, Siri, Berlin, Hyde, Parsons, Joiner & Lawrence (1952) suggest that the body is unable to differentiate between heavy and normal water; but there is some evidence that this may not apply in all situations (Glascock & Dunscombe, 1954; Bowen, 1960). Deuterium oxide has the advantage over tritium oxide that

it is non-radioactive, but the disadvantages that it is difficult to estimate routinely and is expensive. Estimation of a total body water in man with deuterium costs, at present prices, approximately £5; with tritium, the cost is about 1*s*.

Tritium, however, in spite of its radioactivity, is safe to handle and of low toxicity, since it only emits β particles (Brues *et al.* 1952). Nevertheless, until recently it also has been difficult to assay. Previous methods have involved converting to gas and used ionization chambers (Pace *et al.* 1947; Prentice *et al.* 1952; Fallot *et al.* 1957), internal Geiger counters (Wing & Johnston, 1955) and proportional gas-phase counters (Robinson, 1951). But none of these methods is very suitable for rapid and accurate routine analysis of large numbers of samples.

Tritium suffers from the disadvantage that it will exchange with hydrogen other than that present in water. Schloerb, Friis Hansen, Edelman, Solomon & Moore (1950) estimate the labile hydrogen pool to be between 0·5 and 2 % of body weight, and Pace *et al.* (1947) noticed that the virtual tritium space compared with total body water values obtained in rabbits by desiccation procedure was 2–3 % higher. Our own estimations make this pool approximately 4 % of body weight, which is more in keeping with the value quoted by Elkinton & Danowski (1955).

Accuracy of count is dependent on the careful control of several experimental variables. Like Stitch (1959), we also have noted the marked quenching effect of acetone. The Ekco N 612 counter uses a small counting pot. We have noticed up to a 5 % variation in count, using the same tritiated solution in different pots. Each pot was therefore calibrated as regards transmission with respect to one standard pot.

Langham *et al.* (1956) used a trichloroacetic precipitation procedure for human plasma before estimation of tritium. We have compared a trichloroacetic procedure with the one presented in this paper. In our experiments, using eight different plasmas, not only had it no advantage but it reduced the sensitivity of the procedure by about one-third without increasing the accuracy of the recovery.

Our results for total body water are generally slightly higher than those obtained by other workers. Pinson & Anderson (1951), using tritium, reported that the total body water in the rat ranged from 57 to 72 % of the body weight. Flexner, Gellhorn & Merrell (1942), using deuterium, reported mean guinea-pig body water as 65 % of the body weight.

Our figures for virtual tritium space in guinea-pigs are approximately 8 % higher. This may be due to the fact that our animals weighed less than those of Flexner *et al.* (1942) and hence probably had a lower fat content. Another possible reason is that though all the animals appeared normal, at post-mortem a small quantity of clear ascitic fluid was seen.

Our results for total body water in the rabbit are somewhat lower than those of Soberman (1949), who determined antipyrene space. Using deuterium, Schloerb *et al.* (1950) reported that the mean total body water in human beings was 61·8 % of the body weight. Fallot *et al.* (1957), using tritium, reported the value for total body water as 61 ± 4·5 % body weight. Our mean results are higher. The fact that our subjects lived in the tropics may account for some of these differences. Under tropical conditions the biological half-life for tritium was 7·5 ± 0·6 days (mean ± S.E.). This compares with figures of 10 and 9·3 days for the half-life of deuterium (Hevesy & Hofer, 1934; Schloerb *et al.* 1950) and 8–9 days for tritium reported by workers in other latitudes (Fallot *et al.* 1957). Further work is progressing on the effect of climate on water turnover.

SUMMARY

1. A method has been described for the rapid estimation of tritium. The recovery of tritium in certain body fluids by this method has been ascertained and the virtual tritium space has been estimated in a number of mammalian species.

2. In the rat this space was approximately 4 % higher than total body water estimated by a desiccation procedure.

3. The mean biological half-life for tritium in adult male students under tropical conditions was 7½ days.

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THE PERMEABILITY OF THE SQUID GIANT AXON TO RADIOACTIVE POTASSIUM AND CHLORIDE IONS

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In the course of the work described in two earlier papers (Caldwell, Hodgkin, Keynes & Shaw, 1960*a*, *b*) on the link between the metabolism of squid giant axons and the active transport of Na^+ and K^+ ions across their surface membranes, it became necessary to measure the influx of potassium into CN-poisoned axons before and after the injection of energy-rich phosphate compounds. The method first tried was to suspend a well-cleaned axon from a cannula and to take counts in a thin-windowed cell through which inactive sea water flowed, after the axon had been exposed for a short period to a ^{42}K solution. The K influx into *Sepia* axons had been measured with apparent success in a similar fashion (Keynes, 1951), this type of technique having the advantage of allowing each axon to serve as its own control, by exposing it several times to ^{42}K under different conditions. However, it was soon found that when intact squid axons were counted the results were complicated by the presence of substantial quantities of potassium in a superficial region of the axon, which obscured the uptake of ^{42}K by the axoplasm proper. The difficulty was eventually avoided by making measurements on extruded axoplasm, as described elsewhere (Caldwell *et al.* 1960*a*, *b*), but not until a number of experiments had been done on intact axons. The complications encountered have some interest in their own right, and also have a bearing on the results reported by Shanes & Berman (1955). The purpose of this paper is to give a brief account of the preliminary influx determinations, and of a few experiments on the K efflux from axons into which ^{42}K had been injected. The results of some measurements of the influx and efflux of ^{36}Cl are also included.

METHODS

The cell designed for counting intact squid axons is shown in Fig. 1. The cannula into which the axon was tied was mounted in such a way that the axon could quickly be lifted out of the cell and transferred either to a recording cell of the type described by Hodgkin & Katz (1949) for impalement with a micro-electrode or microsyringe, or to a pot containing ^{42}K sea water. Inactive sea water flowed in at the bottom of the cell, and was removed by suction at the top. The position of the axon was carefully adjusted so that its central part

was in contact with a blackened mica window, behind which was a Geiger tube (Mullard Type MX 123). The length of the unscreened part of the window was nominally 12 mm; tests with a speck of ^{42}K solution showed that the counting rate was reduced to half at 6·3 mm each way from the centre of the window. In order to convert uptake measurements in counts/min into absolute units, counts were taken with capillary tubes equal in outside diameter to the axons, filled with a ^{42}K solution of known concentration and held vertically just touching the window. Checks showed that a gap of 100 μ between the tube and the window lowered the counting rate by 3·0 %. (For ^{24}Na, which emits weaker β-radiation, the figure was 8·0 %.) Kinks in the axons may therefore have reduced the counts slightly, but probably by less than 5 %.

For a few experiments the 'flow method' of Hodgkin & Keynes (1955*a*) was used to measure the rate of loss of ^{42}K from an axon which had been exposed for a short period to ^{42}K sea water. The capillary was 16·5 mm long and 1·0 mm in internal diameter.

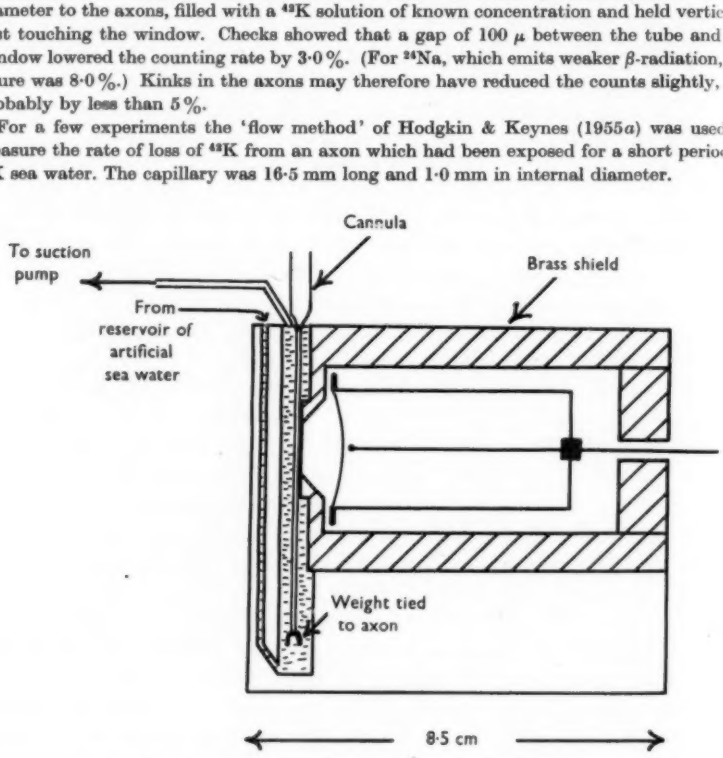

Fig. 1. Diagram, approximately to scale, of the chamber used in counting intact squid axons.

For determination of K efflux, the collecting cell described by Caldwell *et al.* (1960*a*) was used in conjunction with a liquid counter (Mullard Type MX 124). The intracellular potassium was labelled by injecting a 13 or 20 mm column of 1·0 M-K*Cl. For determination of ^{36}Cl efflux, a similar column of 1·0 M-KCl* was injected, and the axon was then tied off at both ends and transferred to a narrow tube containing 0·5 ml. of inactive sea water. After collecting periods of the order of an hour the sea water was changed, the samples being transferred to nickel dishes and dried so that their radioactivity could be determined with an end-window counter. The axon itself was also put on a dish to be counted.

For determination of Cl influx, the 'extruded axoplasm' method of Hodgkin & Keynes (1957) was used. The axons were taken in pairs, uncleaned, one being stimulated at 156/sec throughout the period in ^{36}Cl sea water, while the other was left at rest.

All experiments were done in an artificial sea water the composition of which was: (mM)

NaCl 460, KCl 10·4, $CaCl_2$ 11, $MgCl_2$ 55, $NaHCO_3$ 2·5. ^{42}K samples were treated as described by Caldwell *et al.* (1960*a*). ^{36}Cl samples were received from the Radiochemical Centre, Amersham, as 1·0 M-$K^{36}Cl$ or 1·0 M-$Na^{36}Cl$.

RESULTS

Determination of K influx by counting intact axons

Figure 2 shows the counts taken in an experiment in which a well-cleaned axon was first soaked in ^{42}K sea water for 64 min, and was then cannulated and transferred to the counting chamber (Fig. 1), where it was suspended in a stream of inactive sea water. The puzzling feature of the experiment was the rapidity with which the counting rate decreased, the rate constant for loss of radioactivity being 0·288 hr^{-1}. If all the labelled

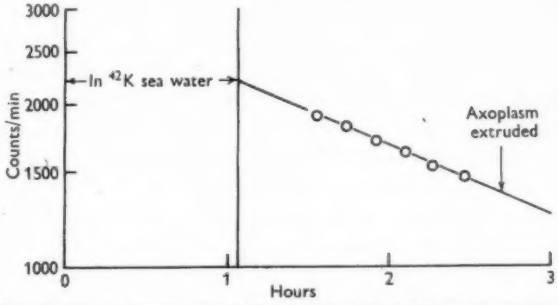

Fig. 2. Counts taken after an axon 812 μ in diameter had been exposed to ^{42}K sea water for 64 min. Temperature 18° C. By capillary calibration an uptake of 1 count/min was found to be equivalent to an entry of 23·3 pmole K/cm axon. At the final arrow, 10·6 mg of axoplasm was extruded from the central part of the axon, and found to contain a total of 3·89 μmole K. In a liquid counter, for which 1 count/min was equivalent to 28·2 pmole K, the axoplasm gave 166 counts/min.mg (all counts are corrected for decay of ^{42}K to the same zero time). Observation of the weight tied to the lower end of the axon showed that the axon did not stretch by more than 1 % during the whole counting period.

K had been in the axoplasm, this rate constant would have implied that the total K efflux was about 625 pmole/cm.^{2} sec (the axon diameter was 812 μ, and its final K content was 385 m-mole/l. axoplasm). The apparent K influx, determined in the usual way (see Keynes, 1951) by extrapolating the counts back to the time of removal from ^{42}K and calibrating with ^{42}K-filled capillaries, was only 24 pmole/cm^2.sec, so that the calculation suggests the occurrence of a net K efflux of 600 pmole/cm^2.sec. However, such a net efflux corresponds to the loss of more than 100 mM-K/hr, and the axon could not in fact have been leaking K nearly as fast as this, since its final K content, some hours after it had been dissected and cleaned, was still almost the same as the figure for fresh axons quoted by Hodgkin (1951). In some other experiments of the same kind the initial value of the

rate constant for loss of radioactivity was even larger, so that there was a still more striking discrepancy between the predicted net loss of K and the amount eventually found in the axoplasm. There was also a marked flattening of the semi-log. plot of counts/min against time (cf. lower curve in Fig. 3), suggesting that the ^{42}K had not entered into a single homogeneous compartment. A similar flattening would, no doubt, have been observed in the experiment of Fig. 2 had counting been continued for a longer period.

The difficulty can be explained by supposing that the fibre behaves as two compartments in parallel, and that in the experiment of Fig. 2 only about half the ^{42}K taken up was actually in the axoplasm, the rest being located in a more superficial part of the fibre, whose exchange-rate constant was much larger than that for the true interior of the axon. Two pieces of evidence support this suggestion. The first is that, as will be seen below, the rate constant for exchange of ^{42}K introduced into squid axons by micro-injection is indeed much less than 0·288 hr^{-1}. The second is that the radioactivity of the axoplasm extruded at the end of the experiment was appreciably less than that calculated for an equivalent volume of the whole axon. From the figures given in the legend to Fig. 2 it turns out that the axoplasm only contributed 1090 counts/min to the extrapolated counting rate of 1370 counts/min at the time of extrusion. Taking the true rate constant for exchange of ^{42}K in the axoplasm as 0·019 hr^{-1} (see Table 1), the contribution of the axoplasm at the beginning of the counting period was probably about 1125 counts/min out of a total 2200 counts/min. Radioactivity was initially lost at a rate of about 200 counts/min in 20 min, whence the rate constant for the 1075 counts/min of the superficial fraction was of the order of 0·54 hr^{-1}. In the period of just over an hour spent in ^{42}K sea water, the specific activity of the superficial fraction would therefore have risen to about 44 % of its equilibrium value, and it can be calculated that the total quantity of superficial K was apparently $5{\cdot}7 \times 10^{-8}$ mole/cm. At a concentration of 400 mM, this amount of K would be contained in a layer roughly 5 μ thick round the outside of an 800 μ axon.

Figure 3 shows the results of another experiment in which the 'flow method' of Hodgkin & Keynes (1955*a*) was used to obtain information about the gradual decrease with time of the rate constant for loss of ^{42}K from an intact axon which had been soaked in radioactive sea water for 90 min. On persevering with the washing away of ^{42}K for 4 hr, the rate constant fell to about 0·026 hr^{-1}, and had thus approached close to the average value of 0·019 hr^{-1} found in the micro-injection experiments. In this experiment the apparent size of the superficial fraction was somewhat smaller than before. From a count of axoplasm extruded at the end of

the washing-out period, the total radioactivity of the 16·5 mm of axon from which ^{42}K was collected could be estimated at each stage of the experiment, and the approximate initial uptake of ^{42}K could then be obtained by extrapolation. It was found that at zero time the axoplasm contributed about 2800 counts/min and the superficial fraction about 1100 counts/min. The exchange rate constant for the superficial fraction was 1·25 hr^{-1}, so that during the uptake period its specific activity would have reached about 85 % of the equilibrium value. Hence the quantity of K in the superficial fraction was estimated as $0{\cdot}94 \times 10^{-8}$ mole/cm axon, corresponding on the same basis as before to a layer about 0·8 μ thick.

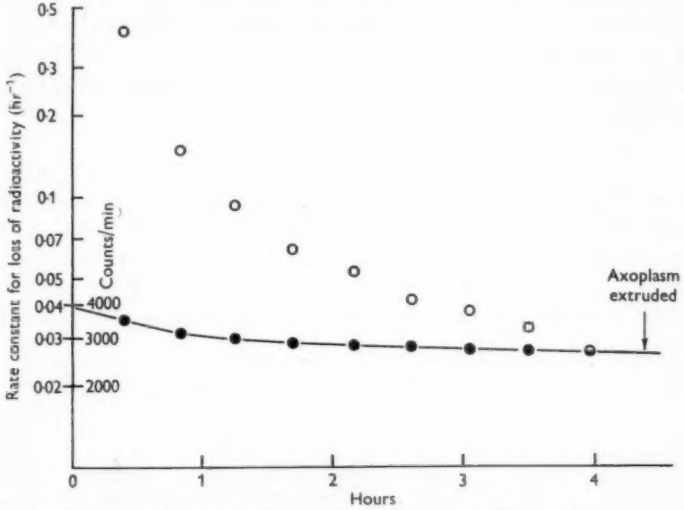

Fig. 3. ○ = the rate constant for loss of radioactivity in inactive sea water from an axon 927 μ in diameter which had been exposed to ^{42}K for 90 min. ● = the total radioactivity in the 16·5 mm length of axon from which ^{42}K was collected. Temperature 11° C. For uptake, 1 count/min was equivalent to 11·9 pmole of labelled K. After the last collecting period, 11·7 mg of axoplasm was extruded from the central part of the axon; this gave a counting rate of 218 counts/min.mg and its K content was 369 m-mole/kg. Zero time corresponded to the moment of removal from ^{42}K. The axon was capable of giving a large spike from end to end throughout the experiment.

In arriving at this figure several simplifying assumptions have been made, and it could well be wrong by a factor of at least two. However, it is important to know the order of size of the superficial fraction, in order to decide whereabouts it might be located.

Because of the existence of this superficial fraction of K, determinations of the K influx by counting intact squid axons are apt to be erroneously

high, the error being greatest when the period of exposure to ^{42}K is short compared with the exchange-time constant for the superficial fraction. The average K influx into cleaned squid axons, measured by counting extruded axoplasm, was $16{\cdot}3 \pm 2{\cdot}2$ pmole/cm^2.sec (S.E. of mean for 5 axons; diameters 766–927 μ; temperature 11–18° C); in these experiments the axons were washed in inactive sea water for 1–5 hr after exposure to ^{42}K, and small corrections were made to allow for the resulting loss of radioactivity from the axoplasm, using a rate constant of 0·019 hr^{-1}. Using uncleaned axons and extruding axoplasm immediately after exposing to ^{42}K, Caldwell *et al.* (1960*a*, *b*) found influxes of 19 ± 2 and 24 ± 3 pmole/cm^2.sec. In contrast, the total influx into 4 axons which were counted intact after spending 5–10 min in ^{42}K sea water, was $33{\cdot}6 \pm 2{\cdot}5$ pmole/cm^2.sec (diameters 680–1068 μ; temperature 16–19° C). Thus only about half the apparent influx into squid axons counted whole represents a genuine penetration of ^{42}K into the axoplasm.

The efflux of ^{42}K introduced by micro-injection

An important check on the conclusions of the preceding section was to label the K in the axoplasm by introducing a column of ^{42}KCl with a microsyringe, and then to measure the rate at which radioactivity emerged from the axon. The results of two experiments on the efflux of injected ^{42}K in which the actions of CN and ATP were investigated have been reported by

TABLE 1. The efflux of potassium from resting and stimulated squid axons loaded with ^{42}K by micro-injection

Expt. no.	Axon diam. (μ)	Temp. (° C)	Rate constant at rest (hr^{-1})	Final K content of axoplasm (m-mole/l.)	Resting K efflux (pmole/cm^2.sec)	Extra K efflux during stim. (pmole/cm^2.imp.)	Calculated diffusion constant for K (cm^2/sec)
4N7	890	18	0·024	320	55	—	—
11N7	912	17	0·014	345	33	—	—
16D7	800	13	0·013	370	26	10·8	$4{\cdot}4 \times 10^{-6}$
17AD7	800	16	0·019	382	39	7·4	$4{\cdot}6 \times 10^{-6}$
17BD7	656	13	0·023	336	39	7·2	$4{\cdot}1 \times 10^{-6}$
Mean			0·019		38	8·5	$4{\cdot}4 \times 10^{-6}$

The length of the injected column of labelled KCl was 13 mm in the first two experiments and 20 mm in the remainder.

Caldwell *et al.* (1960*a*), and the values obtained in these for the rate of loss of radioactivity from resting unpoisoned axons are included in Table 1. It will be seen from the table that the average rate constant for resting axons was 0·019 hr^{-1}. All these measurements were made early in the experiments, when the internal [K] must have been close to about 370 m-mole/l. axoplasm. Using this value in each case, the average resting efflux of potassium was estimated as 38 pmole/cm^2.sec. The net

efflux from cleaned axons at rest was therefore about 22 pmole/cm².sec, corresponding to a loss of some 4 mM-K/hr. Such a rate of loss of K, accompanied by a roughly equivalent gain of Na, is consistent with the results of a number of analyses of squid axoplasm made in the course of this work.

Figure 4 shows the results of an experiment designed to investigate (*a*) how quickly after the injection a steady rate of loss of ^{42}K was achieved, and (*b*) the effect of stimulation on the K efflux. The mid-time of the first

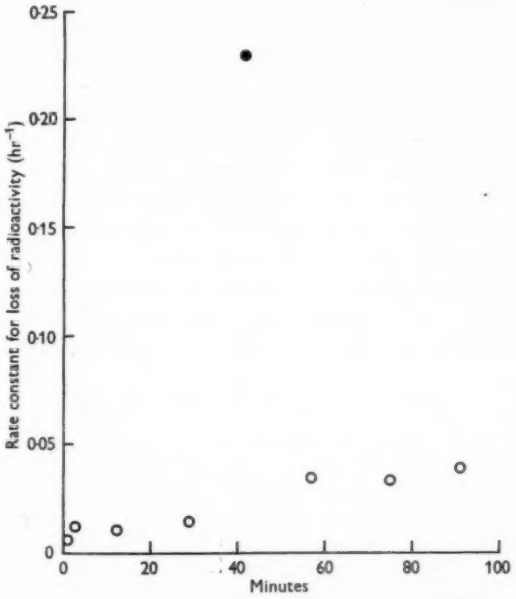

Fig. 4. The rate constant for loss of radioactivity from an 800 μ squid axon into which a 20 mm column of 1·0M-^{42}KCl was injected at zero time. Temperature 13° C. Total radioactivity injected was 45,000 counts/min. During the 11 min collecting period shown as a filled-in circle, the axon was stimulated for exactly 10 min at 50 impulses/sec. For further details see Table 1, Expt. 16D7.

collecting period was 47 sec from the mid-time of the ^{42}K injection, and the ratio of the efflux during this period to the average efflux during the two succeeding periods was 0·53. On the basis discussed by Hodgkin & Keynes (1956), the effective value of the diffusion constant for potassium, calculated as if the whole of the time lag in reaching a steady efflux arose from the slowness of diffusion through the axoplasm, was hence found to be $4{\cdot}4 \times 10^{-6}$ cm²/sec. Two other experiments yielded similar answers. The average apparent diffusion constant for radial movements of K^+ was

therefore less than one third of the value for longitudinal movements of K^+ in *Sepia* axons obtained by Hodgkin & Keynes (1953). It was also somewhat less than the corresponding figure for radial movements of Na^+ ($6{\cdot}5 \times 10^{-6}$ cm²/sec), although in free solution K^+ ions would be expected to diffuse 1·5 times as fast as Na^+. However, it would be unwise to conclude that K^+ ions are unable to move across the interior of the axon as fast as Na^+, because the discrepancy may somehow arise from the presence of a barrier to free diffusion just outside the axon membrane (see Frankenhaeuser & Hodgkin, 1956).

When the axons were stimulated at a frequency of 50/sec there was a marked increase in the efflux of potassium, the average value of the extra K efflux during electrical activity being 8·5 pmole/cm².impulse. The measurements were made at a mean temperature of 14° C; the net loss of K found in *Loligo pealii* axons by Shanes (1954) was 2·9 at 24° C and 8·8 pmole/cm².impulse at 6° C, corresponding to an interpolated value of 5·5 pmole/cm².impulse at 14° C. If in squid, as in *Sepia* (Keynes, 1951), the total efflux of labelled K during an impulse is about 10 % greater than the net efflux, Shanes's figures suggest an extra K* efflux at 14° C of about 6 pmole/cm².impulse. This is close enough to the observed value for *Loligo forbesi* to provide some evidence that the potassium in the axoplasm is uniformly labelled when ^{42}K is introduced by micro-injection.

Determination of labelled chloride fluxes

The influx of Cl^- ions was determined by soaking pairs of uncleaned axons for 20 min in ^{36}Cl sea water, one axon resting and the other stimulated throughout at a frequency of 156/sec. Weighed quantities of axoplasm were then extruded from each axon on to nickel dishes, and their radioactivity was determined with an end-window counter. Table 2 shows that the average chloride influx in resting axons was 12·6 pmole/cm².sec and that, comparing each stimulated axon with its resting control, the increase in influx caused by stimulation was $7{\cdot}2 \pm 2{\cdot}3$ pmole/cm².sec (for which $P = 0{\cdot}01$). The extra entry of labelled chloride resulting from the passage of a single impulse was thus only 0·046 pmole/cm². This value is more than ten times smaller than the certainly less reliable figure obtained by Keynes & Lewis (1951) as a by-product from their measurements of Na and K movements by activation analysis. However, it is not inconsistent with the calculations of Hodgkin & Huxley (1952*b*), which predicted a net Na^+ entry of 4·33 and a net K^+ loss of 4·26 pmole/cm².impulse at 18·5° C, since the difference of 0·07 pmole/cm².impulse could reasonably be attributed to an entry of Cl^-. It is also of the right order to fit with the calculations of Keynes (1951) for the change in anion influx through a membrane whose permeability does not alter when the potential across it varies.

In order to measure the efflux of chloride, 20 mm columns of 1·0 M-K ^{36}Cl were injected into two axons, and the rates of loss of radioactivity were then determined as described under 'Methods'. Owing to the very long half-life of the isotope, the quantity injected gave under 400 counts/min and it was necessary to make the collecting periods rather long (1–2 hr) in order to obtain measurable counting rates. For the first axon, whose diameter was 698 μ, the rate constants for loss of ^{36}Cl during three successive collecting periods were 0·0125, 0·0139 and 0·0157 hr^{-1} (mean = 0·0140 hr^{-1}). For the second axon (796 μ) the rate constants were 0·0120, 0·0095 and 0·0134 hr^{-1} (mean = 0·0116 hr^{-1}). In calculating the absolute value of the chloride efflux some uncertainty arises because of the lack of

TABLE 2. Influx of labelled chloride into resting and stimulated squid axons at 19° C

	Axon resting		Axon stimulated at 156/sec	
Squid no.	Diameter (μ)	Influx (pmole/cm^2.sec)	Diameter (μ)	Influx (pmole/cm^2.sec)
1	524	15·4	531	26·2
2	578	15·0	578	15·0
3	535	7·7	550	11·8
4	542	10·0	531	22·4
5	512	14·7	492	23·5
Mean and S.E.		12·6 ± 1·6		19·8 ± 2·7

reliable figures for the internal [Cl]. If it were taken as 100 m-mole/l. axoplasm, from the analyses of Steinbach (1941) and Keynes & Lewis (1951), allowing for an extra 20 mM added as K ^{36}Cl, the average chloride efflux would be 6·6 pmole/cm^2.sec. However, this value for internal [Cl] is somewhat high to fit with a resting potential in the neighbourhood of 60 mV, and the true figure is possibly only about 50 mM, making the calculated chloride efflux just over 3 pmole/cm^2.sec.

DISCUSSION

Shanes & Berman (1955) determined the resting fluxes of labelled sodium, potassium and chloride in axons from *Loligo pealii*, and arrived at the following values in pmole/cm^2.sec: Na influx 63, efflux 33; K influx 42, efflux 370; Cl influx 14, efflux 87. They pointed out that their figures for sodium were consistent with those for *Sepia* (Keynes, 1951), but they found it difficult to explain their large figures for the effluxes of potassium and chloride. They presented convincing evidence for the existence of a superficial 'X-phase' containing ions in different proportions to the external medium, and estimated that its potassium content corresponded

to a 16·4 μ layer if [K] was taken as 17 mM. They did not, however, make any measurements with extruded axoplasm, nor did they attempt to label the intracellular ions by micro-injection. In the light of our experiments, in which extra evidence from both these techniques was available, it seems clear that most of the troublesome features of their results can be explained as arising from an underestimate of the size of the X-phase. If it had contained somewhat more potassium and chloride than they assumed, they would have obtained flux values which would agree reasonably well with those given here.

It is tempting to identify the superficial X-phase with the Schwann cells and connective tissue which surround giant axons. According to Geren & Schmitt (1954) the thickness of the Schwann cell layer in the axons of *Loligo pealii* is 0·2–1·0 μ. In some unpublished electron micrographs made by Professor A. F. Huxley of permanganate- and osmic-fixed axons from *Loligo forbesi* the Schwann cell layer appears to be 1·5–2·5 μ thick. The greater thickness in *L. forbesi* may either reflect a species difference, or may arise from the greater size of the axons. On the assumption that the superficial phase had a potassium content similar to axoplasm, its thickness was estimated as 5 μ in one experiment (p. 180), and as 0·8 μ in another (p. 181). Both estimates are subject to some uncertainty, but they are clearly of the right order for the identification to be plausible. An additional contribution to the superficial potassium is likely to be made by the cells responsible for laying down the 10–20 μ layer of connective tissue outside the Schwann cells, which are shown in one of Geren & Schmitt's (1954) plates.

If this identification is correct, it is perhaps surprising that a fairly large number of experiments with ^{42}K on *Sepia* axons (see Keynes, 1951; Hodgkin & Keynes, 1955*a*) should have given no hint of the existence of a superficial phase. Electron micrographs of 200 μ *Sepia* axons made by Professor Huxley show a Schwann cell layer about 0·5 μ thick, which might be expected to contain the same amount of potassium relative to that in the axoplasm as the 2·0 μ layer surrounding an 800 μ squid axon. In the *Sepia* experiments the presence of a superficial phase would only have been suspected if there had been (which there were not) marked discrepancies between influx and efflux, or if a consistent flattening of the semi-log. plots of radioactivity in the axon against time had been observed, and a re-examination of the experimental results has confirmed that any such flattening was not obvious. However, it does seem that there was a slight trend towards flattening, since out of 35 groups of counts (many of which comprised only three counts, so that individually they never deviated significantly from a straight line), 14 showed some concavity, 18 showed virtually no curvature at all, but only 3 were convex. This

distribution does differ significantly ($P = 0{\cdot}02$) from a perfectly exponential decline of the counting rate. It must therefore be admitted that the figures published for the potassium fluxes in unpoisoned *Sepia* axons with normal external [K] may all be slightly too high, though to exactly what extent it is impossible to say.

It may be asked whether neglect of a superficial phase could possibly have accounted for the deviations of the potassium flux ratio in *Sepia* axons from the 'independence' relationship which were described by Hodgkin & Keynes (1955*b*). There are at least two reasons for thinking this unlikely. The first is that the deviations in question were too large, and the second is that the flux ratio experiments were all done with DNP-poisoned axons. In each of three squid experiments (not described in the text) in which counts of the ^{42}K in intact axons were taken first unpoisoned, and then after exposure to cyanide, there were indications that the amount of potassium in the superficial phase was substantially reduced by inhibition of metabolism. Thus in each case the flattening of the curve for loss of ^{42}K was prominent at the start of the experiment, but became progressively less so, until in the last group of counts it had always disappeared. Hence the cells which contain the superficial potassium appear to be rather sensitive to metabolic inhibitors, and in the *Sepia* experiments they may well have lost their potassium before the measurements were begun, and so caused no errors in the results. This conclusion is supported by an examination of the data similar to that mentioned in the previous paragraph: out of 21 groups of counts of the ^{42}K in DNP-treated *Sepia* axons, only 3 were concave, 12 showed no curvature, and 6 showed convexity.

It is interesting to consider the resting chloride flux values in relation to electrical data. In the first place, comparison of potassium efflux with chloride influx, using the constant field relation (Hodgkin & Katz, 1949; see also Keynes, 1951), leads to a value of 4·5 for the ratio of P_K to P_{Cl}; a similar calculation for chloride efflux and potassium influx (using the value for CN-treated cleaned axons given by Caldwell *et al.* 1960*a*, in order to avoid errors from the active component of K influx) gives a ratio of 6·5. These ratios are somewhat greater than the approximate figure of 2·2 assumed by Hodgkin & Katz (1949), and serve to emphasize the contrast between the chloride permeability of squid axons, which is confirmed to be less than their potassium permeability, and that of frog muscle, where P_{Cl} has recently been shown (Hodgkin & Horowicz, 1959) to be about twice as great as P_K under normal conditions. Secondly, the chloride flux can be compared with Hodgkin & Huxley's (1952*a*) estimate of 0·26 m-mho/cm^2 for the 'leak' conductance (i.e. the conductance attributed to current carried by ions other than Na^+ and K^+) of the squid axon membrane. Using the flux–conductance relationship derived by

Hodgkin (1951) this conductance would correspond to an ionic flux of 67 pmole/cm^2.sec, that is, to a flux over five times as great as the observed chloride influx. It would therefore seem either that it is wrong to suppose that the whole of the leak conductance is due to the passage of Cl^- ions through the membrane, or that the chloride fluxes are not 'independent' in the sense required for the simple flux–conductance relationship to be valid (see Hodgkin & Keynes, 1955*b*). A similar but slightly smaller discrepancy between chloride flux and chloride conductance has recently been reported for frog muscle (R. H. Adrian, in preparation).

The smallness of the value obtained for the extra influx of chloride during a nerve impulse raises a problem in connexion with Hill's (1950) observations on the volume changes in stimulated axons. In order to account for the swelling that he found, Hill suggested that some of the sodium which moves into a nerve fibre during electrical activity is accompanied by chloride and water, the inward transfer of chloride necessary to fit his results being 1·7 pmole/cm^2.impulse. As the measured entry now turns out to be much smaller than this, some other explanation for the volume change has to be sought, perhaps in terms of a splitting of intracellular molecules during the permeability changes and a consequent inward attraction of water.

SUMMARY

1. Experiments in which cleaned squid fibres were exposed to ^{42}K sea water for a short period and then counted in a stream of inactive sea water showed that much of the radioactivity taken up by the fibre was located in a superficial region. The potassium in this region was exchanged with a time constant of the order of an hour, and its total quantity was estimated as $1–6 \times 10^{-8}$ mole/cm axon. It is tentatively identified with the Schwann cells surrounding the axon.

2. The time constant for loss of ^{42}K introduced into the interior of the axon by micro-injection was about 50 hr. The potassium efflux was increased about 12 times by stimulation at 50 impulses/sec.

3. The average potassium influx into cleaned axons at rest, measured by counting axoplasm, was 16 pmole/cm^2.sec.

4. The average influx of labelled chloride was 12·6 pmole/cm^2.sec when the axons were resting. The extra entry of chloride caused by stimulation was only 0·046 pmole/cm^2.impulse.

5. The time constant for loss of ^{36}Cl introduced into the interior of the axon by micro-injection was about 80 hr.

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PROPERTIES OF REGENERATING NEUROMUSCULAR SYNAPSES IN THE FROG

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Studies of regeneration of motor nerves into muscles (Gutmann & Young, 1944) have shown that a considerable interval of time elapses between the arrival of nerve fibres at the region of denervated end-plates and the earliest restoration of junctional transmission. What follows is an account of some of the local events observed during this stage in the reinnervation of muscle.

METHODS

Regeneration of nerve fibres into frog (*R. temporaria*) sartorius muscles was examined after either complete or partial denervation. The former was achieved by section of the sciatic nerve in the pelvis: the nerve is very easily exposed at a point just rostral to the pyriformis and vastus externus muscles. A stretch of nerve 4–5 mm long was usually removed, but when a longer delay in regeneration was desired, the 7th, 8th and 9th spinal nerves were resected together with the sciatic. In some instances complete denervation was obtained by dividing the sartorius nerve in the muscle itself. Partial denervation consisted in section and removal of one or more of the intramuscular nerve branches (see Miledi, 1960*a*).

At different times after denervation, both sartorii and their nerves were dissected and mounted side-by-side for examination with intracellular micro-electrodes. Acetylcholine (ACh) was applied either diffusely to the two muscles by perfusion of the chamber, or locally to a muscle fibre by iontophoresis through a micropipette. Neostigmine 10^{-6} (w/v) was generally added to the solutions, except when iontophoretic microapplication of ACh was made. The experiments were made from August 1958 to June 1959. For a more detailed account of the experimental procedures see Miledi (1960*a*).

RESULTS

Signs of regeneration before neuromuscular transmission is re-established

Re-establishment of transmission is of course an obvious sign of synaptic regeneration; but as will be shown below this is preceded by a stage in which other signs of regeneration can be detected. A brief consideration of some characteristics of normal and denervated end-plates is therefore relevant.

It has been shown (Katz & Miledi, 1959; Birks, Katz & Miledi, 1960) that junctional transmission and the spontaneous miniature end-plate potentials

(m.e.p.p.s) disappear from a muscle several days after section of its nerve. After a few days of electrical quiescence, subthreshold activity is again detectable at end-plate regions. These m.e.p.p.s found in denervated muscle resemble in many respects those which occur at normal innervated end-plates; but their frequency is much lower and their amplitude distribution is highly asymmetrical.

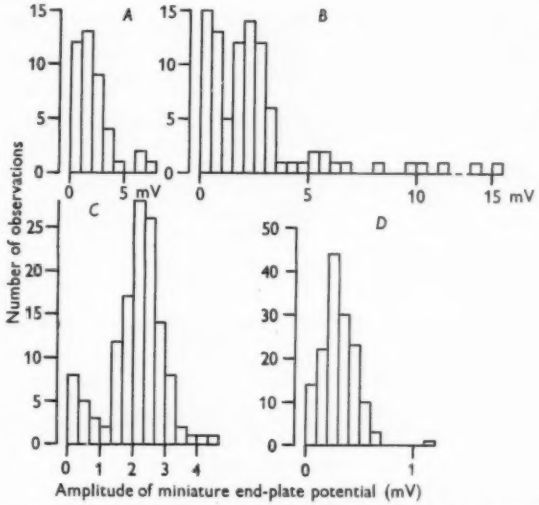

Fig. 1. Amplitude distribution of random spontaneous potentials at end-plates in muscles undergoing neuromuscular regeneration. *A*, *B* and *C* from partially denervated muscles; 41, 51 and 23 days respectively after denervation. *D* from a completely denervated muscle; 109 days. The resting potentials and mean frequencies of m.e.p.p.s were, respectively: *A*, 99 mV, 4/sec; *B*, 90 mV, 0·3/sec; *C*, 86 mV, 1/sec; *D*, 82 mV, 0·6/sec. Prostigmine (1×10^{-6}) was present in *A*, *B* and *C*.

In the present series of experiments, the m.e.p.p. frequency at normal end-plates was usually around 1–4/sec (range, 0·2–20/sec) and their amplitudes had the characteristic bell-shaped distribution (see Fatt & Katz, 1952). On the other hand, the usual rate of m.e.p.p.s at denervated end-plates was about 0·02/sec (range 0·002–0·2/sec) and their skewed histogram resembles that shown in Fig. 1*A* (cf. Birks *et al.* 1960; Miledi, 1960*a*). The rate of discharge of 'normal m.e.p.p.s' is increased by hypertonic solutions and by mechanical irritation at the end-plate region (Fatt & Katz, 1952) while the 'denervated m.e.p.p.s' are not influenced by the latter and may even be reduced by the former procedure (Birks *et al.* 1960). The normally innervated muscle fibre is sensitive to ACh over only a small region in and around the end-plate, while in the denervated fibre the 'sensitive length' is much greater (Miledi, 1960*a*, *b*).

Thus, the conditions prevailing at a typical denervated end-plate at the time of arrival of regenerating fibres may be summarized as follows: (*a*) low-frequency m.e.p.p.s, (*b*) asymmetrical histogram of amplitudes, (*c*) unresponsiveness to hypertonic solutions and (*d*) increased sensitive length and supersensitivity to diffusely applied ACh. Any change towards the normal in one or more of these characteristics was taken as an early sign of regeneration.

Apart from the expected differences in the time of onset of regeneration the results obtained were essentially the same whether denervation had been partial or complete. There was a considerable variation in the behaviour of different end-plates in a given muscle and although individual junctions were not followed through the different stages of reinnervation, the following pattern seems to be established.

Spontaneous electrical activity. The first clear departure from the typical denervated picture outlined above is an increase in the rate of discharge of m.e.p.p.s without any great change in the form of their amplitude distribution. In some cases, e.g. that in Fig. 1*A*, the frequency of m.e.p.p.s was within normal values, but the histogram was still like that of denervated fibres. Since there was a considerable variation in the frequency of m.e.p.p.s at denervated end-plates (see above), it was not possible to decide when a particular fibre showed the earliest sign of regeneration. Nevertheless, denervated end-plates in the frog seldom show m.e.p.p.s at rates higher than 0·2/sec, therefore frequencies higher than that are usually indicative of regeneration.

The time at which the frequency of m.e.p.p.s is increased varies greatly among different end-plates of the same muscle. In extreme cases, even 8–9 weeks after intramuscular section of the pelvic nerve branches there may be no increase in the frequency of m.e.p.p.s at some end-plates while others may already be able to transmit impulses.

The next stage is indicated in Fig. 1*B*: a small group of m.e.p.p.s, whose amplitudes are distributed normally, appears superimposed on the usual asymmetric histogram of denervated end-plates. This group is more obvious in Fig. 1*C* where it makes a major contribution to the histogram. Finally, the amplitude distribution becomes indistinguishable from that found in normal fibres (Fig. 1*D*).

These are the main types of histograms which may be observed in muscles undergoing reinnervation. The probable time sequence at individual end-plates has been inferred from the fact that in muscles whose end-plates have predominantly the simple 'denervation pattern' a few fibres are found in which only the frequency is raised. Conversely, in reinnervated muscles where many fibres transmit impulses, histograms like Fig. 1*D* are a common occurrence.

A rapid series of m.e.p.p.s was frequently observed in regenerating end-plates. These bursts of activity consisted usually of groups of 5–50 m.e.p.p.s. In one case an end-plate examined 118 days after complete intramuscular nerve section showed random m.e.p.p.s at about 0·2/sec for 5 min after the insertion of the electrode. It then suddenly gave bursts of m.e.p.p.s at a mean rate of 3–4/sec for some 3 min before returning to the low frequency

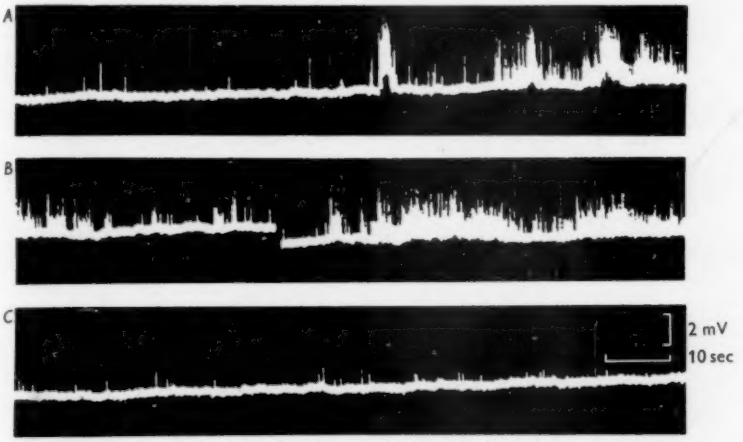

Fig. 2. Bursts of spontaneous potentials at a regenerating end-plate; 118 days after complete intramuscular denervation of the muscle. Resting potential 85 mV. *A*, 5 min after insertion of micro-electrode. *B* is a continuation of *A*. *C* is 2 min after *B*.

(Fig. 2). Another burst of activity occurred 3 min later. It might be suggested that regenerating nerve terminals, like normal ones, respond with such bursts of m.e.p.p.s to the act of prodding with the micro-electrode; however, it seems that frequently the bursts observed at regenerating end-plates are not due to damage caused by the insertion of the micro-electrode, for they were also detected in fibres in which the electrode was first inserted about 2 mm away.

Hypertonic solutions greatly increase the frequency of the spontaneous potentials at normal end-plates (Fatt & Katz, 1952; Furshpan, 1956). Similarly, when the tonicity was doubled by adding sucrose to the bathing fluid the frequency of m.e.p.p.s at regenerating junctions increased even before neuromuscular transmission was re-established (cf. also Birks, *et al.* 1960).

Time course of spontaneous potentials. It is known that the cholinesterase activity at end-plate regions is reduced after denervation (Feng & Ting, 1938; Couteaux, 1955). If the cholinesterase activity remains low for some

time after reinnervation a slow time course of the m.e.p.p.s may be expected. That this occurs is illustrated in Fig. 3, where the slow potential was obtained from an end-plate showing evident signs of regeneration: the m.e.p.p. frequency was 0·09/sec and their amplitude distribution was similar to that in Fig. 1*C*. The muscle had been denervated 69 days previously and neuromuscular transmission was present in some other fibres. Since individual end-plates were not examined throughout their period of

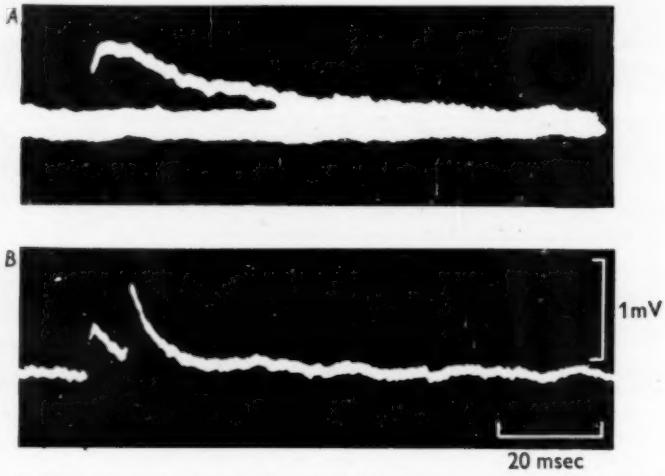

Fig. 3. Time course of m.e.p.p.s. *A*, typical potential from a regenerating junction, 69 days after complete denervation. *B*, from a normal end-plate in the control muscle. Mean frequency of m.e.p.p.s 0·09/sec and 0·7/sec. in *A* and *B* respectively. No prostigmine in the bathing fluid.

regeneration the sequential change in time course of the m.e.p.p.s cannot be determined. Nevertheless, the slow time course of the m.e.p.p.s indicates that the cholinesterase concentration at end-plate regions does not return to its normal level until some time after the arrival of the growing nerve fibre; such a delay has recently been reported by Csillik & Sávay (1959).

Sensitivity to ACh. Complete denervation of a muscle leads to an increase in its sensitivity to diffusely applied ACh. This supersensitivity develops also around denervated end-plates of partially denervated muscle fibres (Frank, 1959; Miledi, 1959, 1960*a*).

If the nerve is allowed to grow into a denervated muscle the supersensitivity eventually disappears (cf. also Hines, Thomson & Lazere, 1942; Emmelin & Nordenfelt, 1959). The particular stage of regeneration at which supersensitivity disappears from individual end-plates was not

ascertained. It was, however, clear that an end-plate may show evident signs of regeneration and still be supersensitive. This is illustrated in Fig. 4 obtained from a muscle 41 days after partial denervation of only approximately one half of the muscle fibres (see diagram in Fig. 4). The proximal tibial branch was not cut, so that this part of the muscle served as a control. The denervated and more sensitive end-plate is undergoing regeneration, as is evidenced by the normal frequency of m.e.p.p.s (4/sec). The amplitude distribution of these potentials was still of the 'denervated' type and is shown in Fig. 1*A*.

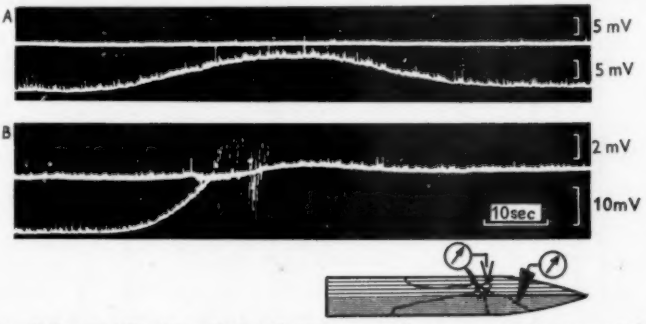

Fig. 4. Effect of diffusely applied ACh on a normal (top traces) and a regenerating (bottom traces) end-plates, 41 days after partial denervation. ACh perfusion was started near the beginning of the traces, in concentrations of 1×10^{-9} (*A*) and 1×10^{-8} (*B*). This last concentration produced vigorous twitching in the half of the muscle which had been partially denervated (see diagram).

Resting potentials were 96 and 99 mV in normal and regenerating end-plates respectively. The interrupted line in the diagram indicates the position of the cut nerve branches. The solid triangle indicates the approximate location of the micro-electrode in the regenerating end-plate.

Even 4 months after complete intramuscular denervation a muscle may still be supersensitive. This is not surprising, since on examination some end-plates are found which do not show any signs of regeneration, and many more which have abnormal histograms and do not transmit impulses although the rate of m.e.p.p.s is nearly normal.

Length of muscle fibres sensitive to ACh. The normally innervated muscle fibre is sensitive to iontophoretic application of ACh only in the region of the neuromuscular junction and its immediate surroundings (Miledi, 1960*b*). The 'sensitive length' of normal frog sartorius fibres (as defined by Miledi, 1960*a*) is about 1·1 mm, while the length of the terminal arborization of the nerve fibre ranges from about 50 μ to 500 μ (Kühne, 1887; Couteaux & Tazi, 1952). After denervation the sensitive length increases progressively until the whole muscle fibre surface becomes sensitive to ACh (Axelsson & Thesleff, 1959; Miledi, 1959; 1960*a*).

On examining fibres whose neuromyal junctions were in the process of regeneration—i.e. fibres in which the frequency and amplitude distribution of m.e.p.p.s was about normal—it was found that the sensitive length returns to near normal values before neuromuscular transmission is re-established. Furthermore, in a few cases the sensitive length was within

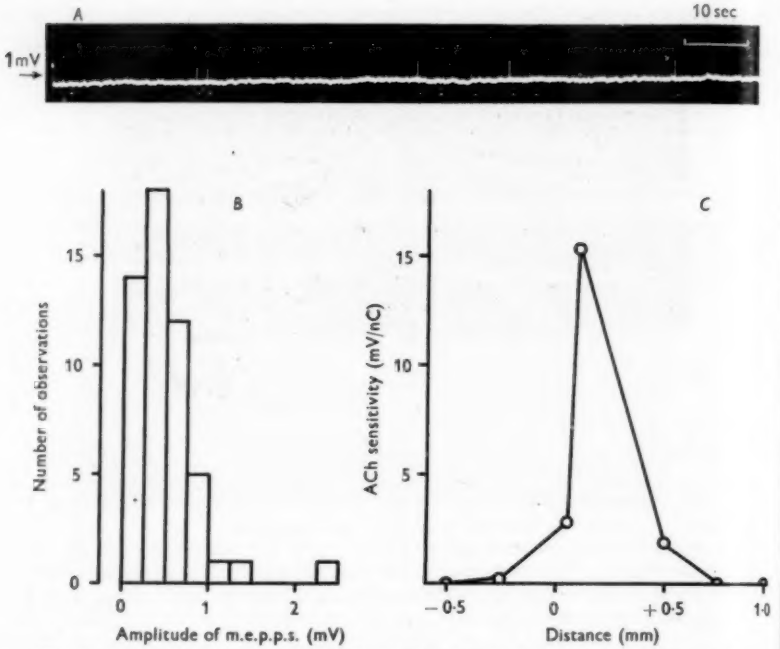

Fig. 5. Spontaneous activity and ACh-sensitive length of an end-plate in a muscle 109 days after pelvic section of the sciatic nerve.

A, examples of m.e.p.p.s. The dots mark the appearance of a m.e.p.p. as observed with a fast time base on a monitor tube. *B*, amplitude distribution of the m.e.p.p.s. *C*, distribution of ACh-sensitivity along the muscle fibre. The abscissae indicate distances from the recording electrode at which ACh was applied.

the normal range of values even though the frequency and amplitude histogram of the m.e.p.p.s had not yet clearly departed from that usually found in denervated end-plates.

For example, a sartorius muscle was examined 109 days after section of the sciatic nerve. Had there been no reinnervation, the fibres in this muscle would have been sensitive to ACh over their entire length (cf. Miledi, 1960*a*). Nevertheless, two junctions were found in which the m.e.p.p. frequency and histogram (Fig. 5*A* and *B*) were typical of dener-

vated end-plates while the sensitive length was like that of normal muscle fibres (Fig. 5*C*). Stimulation of the nerve had no effect on these junctions. There was presumably at least one other end-plate on the same fibre, because of the extensive multiple innervation of frog sartorius (cf. Katz & Kuffler, 1941); but if this had become reinnervated it did not transmit propagating impulses. Some fibres in the same muscle had normal sensitive lengths and normal m.e.p.p.s but did not respond to nerve impulses, while in others regeneration had gone further so that stimulation of the nerve evoked muscle action potentials and twitches.

Onset of neuromuscular transmission

As previously mentioned (cf. also Birks *et al.* 1960) during regeneration it was possible to find some end-plates in which the m.e.p.p.s had returned to the normal pattern whilst neuromuscular transmission had not yet been established.

The inability of regenerating synapses to transmit impulses is not due to accidental damage caused to the nerve fibres during dissection or insertion of the micro-electrode. This possibility was ruled out by the fact that in some muscles no twitch was seen on stimulation of the spinal nerves even before dissecting out the muscles. Later on these same muscles were found to have regenerating end-plates.

Regenerated junctions which transmitted impulses had normal m.e.p.p.s except for one end-plate in a partially denervated muscle (21 days) which had spontaneous potentials at a rate of 0·3/sec and a histogram like that shown in Fig. 1*B*. A further point of interest is that subthreshold end-plate potentials (e.p.p.s) were not observed in regenerating end-plates (unless blocking agents were applied). In other words, the junctions either generated normal e.p.p.s and spikes or did not respond at all to nerve stimulation. This suggests that once a nerve impulse liberates ACh the quantity released is not very different from normal.

Although 'transmitting' regenerated end-plates had a normal pattern of spontaneous activity, they still differed from normal junctions in the latency of their responses to nerve stimulation. This is illustrated in Fig. 6 (cf. also Fig. 7) which shows records of e.p.p.s obtained simultaneously from two end-plates in the same curarized muscle. The muscle had been partially denervated 3 weeks before by section of one tibial branch. It can be seen that the latency of the e.p.p. in the regenerated fibre (bottom record) is greater than that of the non-denervated end-plate, in spite of the fact that the conduction distance was about 2 mm larger for the latter—i.e. a difference of 4 msec for only approximately 2 mm of regenerated nerve fibre. A similar difference was found if regenerated end-plates were compared with end-plates in the normal control muscle.

When the regenerating nerve fibres had to travel a long distance to reach

the end-plates, for instance, after section of the nerve in the pelvic region, the latency of the responses to nerve stimulation was considerably greater than that shown in Fig. 6. For example, in a muscle examined 80 days after denervation the latency of the e.p.p. in some regenerated synapses was 15–20 msec. The conduction distance was about 25 mm, which gives an *average* conduction velocity of 1–2 m/sec in the regenerated nerve fibres.

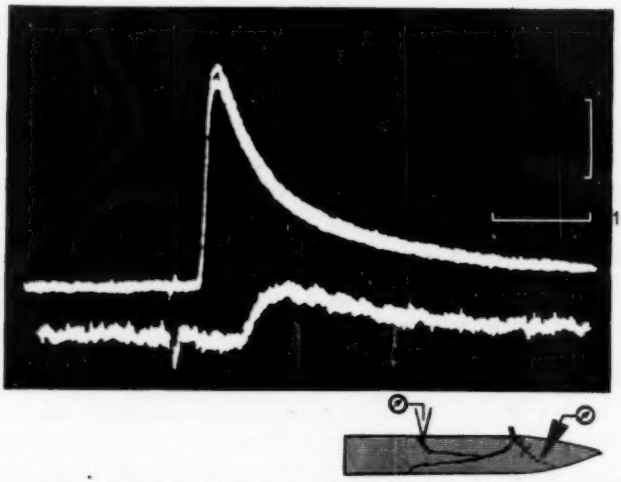

Fig. 6. End-plate potentials in a normal (top trace) and a regenerated (bottom trace) end-plate of a partially denervated muscle. Distances are: 21 mm from stimulating electrode on nerve to 1st branching; 2·7 mm from nerve bifurcation to electrode in the regenerating end-plate (solid triangle in diagram), and 4·9 mm from bifurcation to normal end-plate. Resting potential was 80 mV at both end-plates. The voltage calibration represents 3 mV in normal and 1 mV in regenerating end-plate; tubocurarine concentration, 6×10^{-6}.

A further difference between normal and regenerated transmitting synapses lies in their response to repetitive nerve stimulation. Some end-plates would fail to produce e.p.p.s if the nerve was stimulated at frequencies greater than 0·1–0·2/sec. This failure appeared abruptly as an all-or-none phenomenon and resembled the presynaptic failure of propagation of nerve impulses which occurs near normal rat neuromuscular junctions during repetitive activation (Krnjević & Miledi, 1958). Presynaptic blockage of impulses may be seen in normal frog junctions (cf. also Kostiuk, 1958), but it always appeared more readily, and at low rates of stimulation exclusively, at the regenerated synapses.

Repetitive activation of the normal nerve terminal leads to a progressive decrease in the amplitude of the e.p.p., mainly as a result of a

reduction in the amount of ACh released by the nerve impulse (unpublished). This fall in amplitude was usually greater in regenerated end-plates (cf. Fig. 7*B*). Thomson, Morgan & Hines (1950) found that the contraction produced by repetitive nerve stimulation fell more rapidly in regenerating than in normal muscle. Such a phenomenon may be satisfactorily explained by the readiness with which presynaptic block occurs in regenerating nerve fibres plus the more marked decrease in the amplitude of the e.p.p.

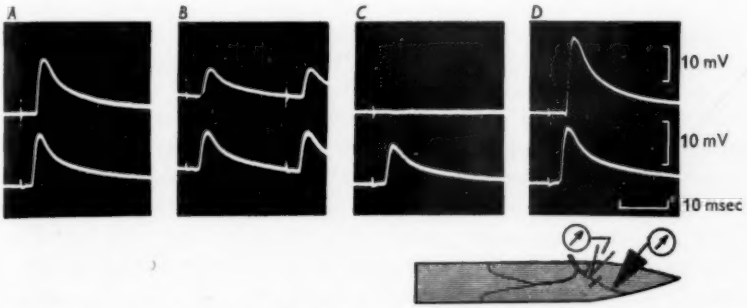

Fig. 7. Tetanic and post-tetanic effects at a regenerated (top trace) and a normal end-plate (bottom trace), 9 days after section of the tibial nerve branch. Resting potentials: 95 mV in regenerated and 82 mV in normal end-plate. *A*, control potentials. *B*, during repetitive stimulation (50/sec for 10 sec). *C*, 15 sec after the end of the tetanus. *D*, 30 sec later. Tubocurarine concentration 3×10^{-6}.

Post-tetanic phenomena at regenerated end-plates also differ from normal. The well known post-tetanic increase in the amplitude of synaptic potentials was greatest at regenerated junctions. The relatively complicated effects of tetanic stimulation are illustrated in Fig. 7. The top records are from a regenerated synapse while the lower ones correspond to a 'normal' one in the same muscle (see diagram). The nerve was stimulated once every 15 sec and the control e.p.p.s are shown in Fig. 7*A*. Stimulation at 50/sec was then applied for 10 sec, and Fig. 7*B* illustrates the potentials shortly before the end of the tetanus; notice the greater fall in amplitude at the regenerated junction and that there was no presynaptic block during the tetanic stimulation.

The first test stimulus after the end of the tetanus (Fig. 7*C*) produced in the normal fibre a still depressed e.p.p.; and no response at all in the regenerated end-plate. This complete failure is probably due to presynaptic block of nerve impulse propagation, for the third and subsequent test stimuli (Fig. 7*D*) suddenly produced an e.p.p. which was 1·4 times greater than the control; the potentiation lasted about 5 min. On the other hand, there was practically no potentiation of the normal e.p.p. A 15 sec tetanus

(not shown in Fig. 7) was followed by a 1·3 min presynaptic block of the regenerated fibre and by a greater and longer-lasting post-tetanic potentiation: the maximal e.p.p.s were 1·9 and 1·3 times as large as their controls in the regenerated and normal end-plates respectively.

The appearance of complete presynaptic block on reducing the frequency of stimulation is a phenomenon which can also be observed in normal junctions in the rat diaphragm (unpublished experiments with Dr K. Krnjević). It also occurs at normal junctions in the frog sartorius, but in both cases it is usually necessary to prolong the tetanic stimulation until an intermittent presynaptic block develops before reducing the frequency of stimulation.

The site of reinnervation

An attempt was made to find out whether regenerating fibres grow into old end-plate regions or form new ones. Since the neurilemmal tubes remain after axon degeneration, they may guide the growing fibres to the old end-plates; an effort was therefore made to remove these tubes. For this purpose a few muscles were denervated by dividing the nerve at its entry into the muscle and removing its intramuscular nerve branches as completely as possible. The branches were dissected in the deep surface of the muscle and when dissection could not be pursued further without gross damage to the muscle fibres the nerves were 'evulsed'. In this way there was not much nerve left which could guide regenerating fibres to old end-plates in superficial muscle fibres.

It is worth considering at this point the location of the main end-plate foci in the normal sartorius. With minor variations the principal nerve branches are as illustrated in Fig. 7. In some muscles there is an extra nerve branch which innervates some muscle fibres at the edge opposite the nerve entry (cf. Fig. 4). The two pelvic branches and that near the nerve entry are clearly visible, especially at the edges where end-plates can be located quite accurately. Parallel to the tibial nerve branch there is usually a long succession of end-plates. If the micro-electrode is inserted in these regions some 90% of the impalements are found to be near end-plate regions (cf. Birks *et al.* 1960).

The muscles were examined 4 months after denervation and it was immediately noticed that the regenerated fibres did not form bundles, i.e the gross pattern of innervation was not reconstituted. After staining with osmic acid the nerve was found to fan out immediately after its entry into the muscle, a situation similar to that described by Aitken (1950) for rabbit muscle. Nevertheless, on examining the different regions with the micro-electrode spontaneous electrical activity, indicative of regeneration, was found only in the normally expected places. It is particularly important that regenerated junctions in the pelvic edges were found at their usual site some 8–15 mm away from the nerve entry. In spite of the fact that in these muscles no nerve branches were visible to aid in the location of end-plate foci the number of successful insertions, using the 'normal' map of end-plate distribution, was quite high. In one muscle 22 out of 26 inser-

tions were at end-plates all showing m.e.p.p.s of normal frequency and all in expected places.

There are other indications that regenerating fibres go to the old synaptic regions. Thus one may find an end-plate focus in which some fibres conform to the characteristic denervated pattern of low-frequency m.e.p.p.s and asymmetrical amplitude distribution, intermingled with junctions showing signs of regeneration. Furthermore, m.e.p.p.s were never found when insertions were made in regions in which no end-plates could normally be expected. All these findings suggest that the growing nerve fibres do not just form end-plates anywhere along the muscle fibres but grow towards the site of the denervated end-plates.

DISCUSSION

Several days after cutting the motor nerve the Schwann cell replaces the axon in its synaptic position. It has been suggested that it is this Schwann cell which is responsible for the release of the ACh packets which cause the spontaneous potentials observed at denervated end-plates. The potentials produced by these packets have a skewed amplitude distribution while those produced by ACh released from axon terminals have a normal distribution (Birks *et al.* 1960). During regeneration histograms with both characteristics are obtained. This is probably a consequence of the existence of *two sources* of ACh. Presumably at this stage both the Schwann cell and the regenerating nerve fibre make close contact with the muscle fibre and liberate ACh; a close apposition is indicated by the relatively fast time course of the m.e.p.p.s. During more advanced regeneration, when a normal histogram prevails, the Schwann cell either ceases to release ACh or else it is displaced from the chemosensitive muscle surface by the growing nerve fibre in such a way that if it still liberates ACh this could pass undetected.

It was shown that regenerating nerve fibres make synaptic contact with muscle fibres in a region near the old end-plates, even when the intramuscular nerves are resected. The method of localization did not allow a more precise determination of the site of contact; nevertheless the following point should be considered. Spontaneous potentials in denervated muscle are seen *only* at the end-plate; therefore if the interpretation of the composite histogram of regenerating end-plates is correct and indicates synaptic release of ACh from both the growing nerve fibre and the Schwann cell, then we are forced to conclude that the regenerating axons grow not merely to a place near the old end-plate but precisely to the old synaptic region.

This conclusion differs from that of Gutmann & Young (1944) and Aitken (1950) who state that end-plates may be formed anywhere on the muscle

fibre. The discrepancy may be due to differences in species (rabbit as compared with frog) though Tello (1907), also working on rabbits, mentions that in most cases regenerating fibres grow to old end-plates. In view of the new physiological evidence, further experiments on mammals are clearly needed to ascertain whether new synaptic regions can be formed in uninjured muscle fibres.

Since in the frog regenerating fibres go to the original synaptic regions, the question arises as to how the nerve fibres find their way. If we bear in mind that a 'neurotropic' substance may be involved in the sprouting of normal nerve fibres of partially denervated muscle (Edds, 1950; Hoffman, 1950), then the simplest explanation would be the release of a neurotropic substance from the denervated end-plate or its Schwann-cell overlay, thus causing the fibres to grow to the original end-plate. It is also possible, of course, that the nerve fibres are able to establish synaptic contact with the muscle only at the end-plate region, in which case the regenerating fibres would wander about until they come in contact with an end-plate site. Whichever the mechanism involved, it now seems at least clear that the end-plate region has a special neural affinity.

The distinctiveness of the end-plate region remains even after several months of denervation. Tello (1907), Tower (1939) and Guttmann & Young (1944) could distinguish it histologically, and the synaptic folds are clearly recognizable with the electron microscope (Birks *et al.* 1960), but the identity of the factor responsible for the neuro-affinity awaits further research. As for neurotropism, Tello (1907) has suggested that the end-plate nuclei attract regenerating nerve fibres. Again, Tello (1917) and Couteaux (1941) in their interesting studies on the genesis of neuromuscular connexions reached the same conclusion to account for their constant finding of nuclei at the site, where during development the nerve fibre first comes in contact with the muscle fibre.

Csillik & Sávay (1959), using histochemical methods, claim that the cholinesterase activity of the denervated subneural apparatus exerts a neurotropic action. They further state that the subneural apparatus eventually disappears and that during regeneration a new one is formed. Nevertheless, their results may be interpreted very differently. For example, they show pictures of regenerating endings in which synaptic folds are revealed by the cholinesterase stain. This may mean that synaptic folds are formed in a region previously devoid of them, or else, as seems more likely, it simply indicates the re-formation (or concentration) of cholinesterase in the old synaptic folds.

Transmission of impulses through regenerated end-plates does not start immediately after the growing axon makes synaptic contact with the end-plate region. The failure to transmit is clearly not due to lack of ACh in

the axon terminal, since m.e.p.p.s are produced. It could be that the nerve impulse invades the terminal but fails to liberate ACh, but since the ending reacts 'normally' to certain agents, such as hypertonic solutions and mechanical irritation, this seems most unlikely. Presumably what happens is that the impulse fails to invade the ending because at this stage the regenerated fibre is unable to propagate impulses. The long latency observed when transmission is re-established is probably due to the slow conduction of regenerating fibres (Berry, Grundfest & Hinsey, 1944, Sanders & Whitteridge, 1946). A very low velocity is to be expected in view of the very small diameter of regenerating fibres (Cajal, 1928; Tello, 1907; Hoffman, 1955).

It is becoming increasingly evident that motor nerve fibres exert upon muscle influences which are very different from the mere initiation of contractions. Buller, Eccles & Eccles (1960*a*, *b*) have found that nerve fibres control the differentiation of muscle into slow and fast muscles; and that such a control is probably exerted independently of neuromuscular impulses. A clear dissociation of a neural influence from muscular activity is found in the increased sensitivity of partially denervated muscle fibres (Miledi, 1960*a*), thus demonstrating a discrete neural influence which controls the number and spatial spread of ACh receptors in the muscle fibre membrane. It is the operation of that influence which on reinnervation again restricts the ACh sensitivity to a small region of the muscle fibre.

The measurements of the length of muscle fibre sensitive to ACh are of importance in connexion with the nature of the 'receptor-controlling factor' and its possible relation to the transmitter substance. The fact that the sensitive length returns to normal values before transmission is restored indicates that the receptor-controlling factor is liberated *independently* of nerve impulses. The factor might still be associated with the spontaneous liberation of the transmitter responsible for the m.e.p.p.s; but this is probably not the case, since normal sensitive lengths were found in a few regenerating end-plates in which the frequency of the m.e.p.p.s was no higher than that in denervated end-plates. It seems therefore that the receptor-controlling factor is different from the transmitter and is liberated separately; but what it is and whether it has actions other than regulating the receptor units are questions at present unanswerable.

SUMMARY

1. Regenerating neuromuscular synapses have been examined, with intracellular electrodes, after partial or complete denervation of frog sartorius muscles.

2. The frequency and amplitude distribution of the spontaneous potentials return to normal before neuromuscular transmission is restored.

3. The length of muscle fibre sensitive to acetylcholine is reduced before transmission is re-established.

4. In response to a nerve stimulus, pure end-plate potentials were not observed in regenerating junctions. The end-plate potentials were either large enough to generate muscle action potentials or did not appear at all.

5. 'Transmitting' regenerated end-plates differed from normal in that the latency of their responses was greater, presynaptic failure of propagation occurred more readily and post-tetanic potentiation was more pronounced.

6. Regenerating nerve fibres grow to the regions of the denervated end-plates.

7. It was concluded that the neural factor which controls the number and spread of acetylcholine receptors in the muscle fibre is independent of nerve impulses. The evidence further suggests that the 'receptor-controlling factor' is not the transmitter substance and that it is liberated independently of it.

I am indebted to Professor B. Katz for much helpful discussion. I also wish to thank Mr J. L. Parkinson for continuous help and Mr A. C. Downing for a constant supply of micro-electrodes.

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SINGLE-UNIT ACTIVITY IN THE LATERAL GENICULATE BODY OF THE CAT

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All impulses of retinal origin going to the visual cortex are relayed through the lateral geniculate body. Both its complex anatomical structure (O'Leary, 1940; Clark, 1942; Walls, 1953) and the functional properties of its synapses, as revealed by electrical stimulation of the optic nerve (Bishop & Evans, 1956; Bishop, Burke & Davis, 1958, 1959; Bishop & Davis, 1960), suggest that this nucleus provides a site for integrative mechanisms.

Brindley (1960) and Crescitelli (1960) have recently discussed the role of the lateral geniculate body. Hubel (1960), recording the responses of single geniculate units in conscious, unrestrained cats, found that the activity of the lateral geniculate body is modified by arousal; the organization of the receptive field, however, as seen in the retina, undergoes no change until the impulses reach the cortex.

In the present study, of which preliminary communications have appeared (Erulkar & Fillenz, 1956, 1958), the responses of single units in the lateral geniculate body of cats anaesthetized with sodium pentobarbitone to diffuse light stimuli were studied. Variation in the light intensity and different combinations of binocular stimulation revealed the occurrence of some integration at the level of the lateral geniculate body.

METHODS

Eighteen cats were used. Each cat was initially anaesthetized with sodium pentobarbitone 42 mg/kg (Nembutal; Abbott Laboratories). Subsequent maintenance doses of 14 mg Nembutal were administered either intramuscularly or intravenously when necessary.

When the cat was deeply anaesthetized a tracheal cannula was inserted. The brain was exposed and the cerebral hemisphere overlying the lateral geniculate body was removed by gentle suction. The nictitating membranes were cut away, and the cat's pupil was dilated by the application of a few drops of 1 % atropine to the cornea. In some experiments the eyeball was fixed by stitching a wire ring to the corneoscleral junction. Following these procedures the animal was placed in a stereotaxic holder and transferred to a light-proof box.

A small tungsten-filament lamp, operated from a 6-V battery, provided the source of illumination. The beam of light which was in the direction of the visual axis was focused

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on to the cornea by a system of lenses. The angle subtended at the eye by the source of light was approximately 16° and the area of retina illuminated by direct, as distinct from scattered, light was 2–3 mm in diameter. The stimulus intensity could be varied by the interposition of an Ilford neutral gelatine wedge filter, calibrated to a range of 4 log. units. The stimulus was signalled, as a deflexion of the lower trace of the oscilloscope, by means of a phototransistor placed in the path of the light beam, and the stimulus duration, ranging from 0·5 to 10 sec, was controlled by a camera shutter.

For binocular stimulation two light sources identical in principle with that used for monocular stimulation were used. Precautions were taken in this series of experiments to screen one eye from the other, so that one light source could not stimulate both eyes. Reflexions from the wall of the box in which the cat was placed were minimized by covering the walls with black paper. The temporal relationships of the two stimuli were calculated from the signals on the records. In all experiments the light beam was directed at the area centralis of the cat's retina.

The micro-electrodes consisted of Wollaston wire encased in glass capillary tubing. The tip diameters of these electrodes ranged from 2 to 10 μ. Changes in potential between these electrodes and an indifferent electrode attached to the scalp were led to a 4-stage capacity-coupled Grass P-4A pre-amplifier. The potentials were then led from the pre-amplifier through a power amplifier to a loudspeaker, and also to a Cossor 1035 double-beam oscilloscope, where they were photographed.

In each experiment the cat was first allowed to dark-adapt for 2 hr. The electrode was then introduced into the lateral geniculate body under direct vision. During each puncture the eye was stimulated with light flashes until a regular well-defined unitary response was obtained. Four to five flashes at this intensity were then given and the responses photographed. Intervals of 25 sec were allowed between flashes. The stimulus intensity was then changed, and another series of four to five flashes given. Light intensities were chosen at random and at least 1 min was allowed between successive intensity series, during which time the cat was in complete darkness. Units could be held for periods up to 2 hr, and many of the ones on which detailed measurements were made we held for 30 min or more.

In some initial experiments we varied the duration of the stimulus, keeping the intensity constant. We found that the duration of the flash can be an important variable, but that there is a 'critical duration' (Lennox, 1958) above which the characteristics of the response do not alter. The value of this critical duration varied from one unit to another. We have always used flashes of at least critical duration in later experiments.

RESULTS

The activity of 165 units in the lateral geniculate body was recorded; 84% of these units showed impulse activity in complete darkness. The frequency of this discharge varied considerably not only between different units, but also for the same unit during the period of recording, which in some cases was as much as 90 min. No unit firing in darkness ever showed a regular frequency over a sustained period of time: the activity consisted of brief, high-frequency bursts of impulses, or of trains of impulses at a low frequency.

Of the 165 units only 19 did not respond to illumination of one eye, but since only a fraction of the retina was illuminated the receptive fields of these units may have failed to be stimulated. The remaining units showed a variety of response patterns: 57% were 'on–off' responses, 21% were

'on' responses, 6% were 'off' responses and in 5% there was suppression of the spike discharge during illumination. The responses consisted of either brief, high-frequency bursts of spikes (called 'bursts') or longer, low-frequency trains of spikes (called 'trains'). In most 'on–off' responses both responses were of the same type, i.e. the 'on' and 'off' responses were both either bursts or trains; but in some cases the 'on–off' responses were of mixed type. Changes in intensity sometimes produced a change from one response type to the other. Also in 'on–off' responses the 'on' and 'off' responses often had different thresholds, and therefore the response pattern was not constant over the entire intensity range. For these reasons a rigid classification of units into response types was not possible.

Some units responded to illumination of the ipsilateral, others of the contralateral, eye. During a number of vertical electrode penetrations we found two zones of units responding to contralateral eye stimulation separated by an intermediate zone of units responding to ipsilateral eye stimulation. The response characteristics measured in this study were response latency, response duration, spike number and frequency pattern. In a succession of identical light flashes, the first response was usually optimal, i.e. had the shortest latency, highest frequency, greatest spike number, etc. Subsequent light flashes with intervals of 25 sec produced diminishing responses. Two or three minutes in complete darkness were required to restore the response characteristics to their original level.

Monocular stimulation

Changes in the intensity of the light stimulus produced changes in a number of response characteristics. The values of the response characteristics were plotted against the log. intensity of the stimulus. In most cases the points so plotted showed a fairly clear-cut relationship, although in a few cases they appeared to be scattered at random.

An intensity range of 4 log. units was available. The graphs plotting log. intensity against response latency were of two kinds: in one the latency shortened and in the other it lengthened with increasing stimulus intensity. The first type of response (Fig. 1), which will be called 'latency-decreasing', was found in 44 'on' and 32 'off' responses. The second type of response (Fig. 2), called 'latency-increasing', was found in 5 'on' and 8 'off' responses. In units giving 'on–off' responses only one part of the response, either the 'on' or the 'off', was of the latency-increasing type, the other being latency-decreasing. In both types of graph the curve flattened off at higher intensities, but the scatter of the latency figures was greatest when the latencies were longest.

We found a considerable range of latency figures, both for different

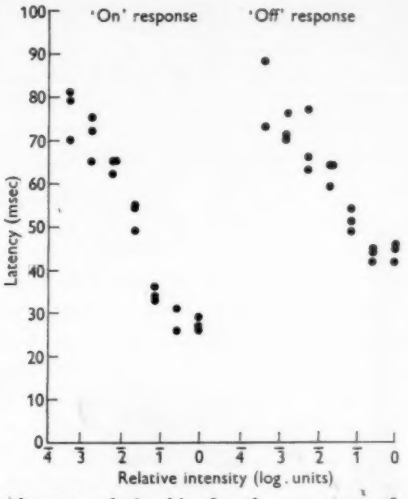

Fig. 1. Intensity–latency relationship for the responses of a unit showing shortening of latency with increase in stimulus intensity. Each point on the graph represents one response latency.

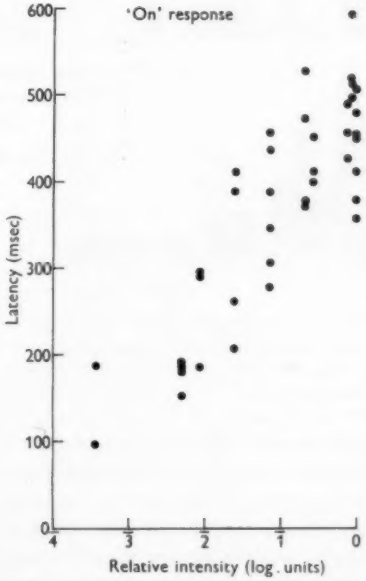

Fig. 2. Intensity–latency relationship for the responses of a unit showing lengthening of latency with increasing stimulus intensity.

units at a given light intensity, as well as for a given unit over the available 4 log. units of intensity range.

Figure 3*a* and *b* shows the range of latencies for 'on' and 'off' responses. We chose the maximum intensity of our light stimulator, which was 3–4 log. units above threshold for most of the responses, and at which most of the intensity–latency curves showed the flattening mentioned above. The

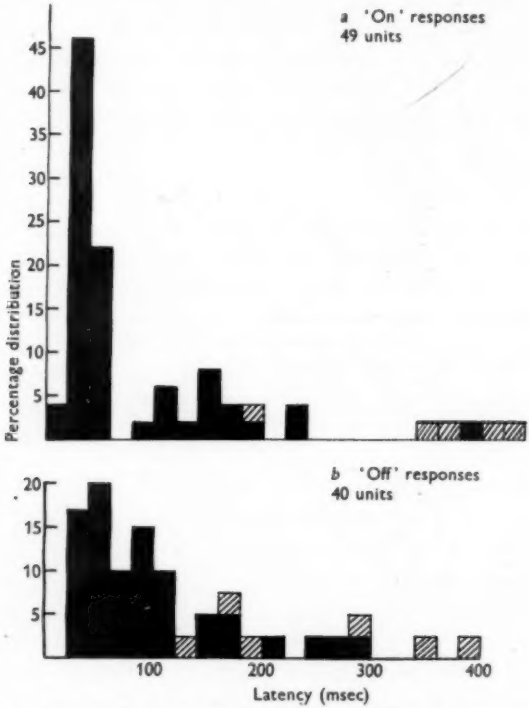

Fig. 3. Histograms showing percentage distribution of latencies for 'on' and 'off' responses. Latency figures are mean response latencies for each unit at maximum stimulus intensity. Black columns are latency-decreasing and cross-hatched columns latency-increasing units.

mean latency at this intensity can therefore be regarded as the minimum latency for the latency-decreasing responses and the maximum latency for the latency-increasing responses. The latter, indicated by cross-hatched columns, make up only a fraction of the long-latency responses.

The change in latency with intensity varied considerably for different units. We calculated the difference between mean latency at threshold and at maximum stimulus intensity for all the responses and compared the

different response types. Latency-decreasing 'on' responses showed a shortening of latency between threshold and maximum stimulus intensity of 10–234 msec; latency-increasing 'on' responses showed a lengthening of latency of 18–293 msec over the same intensity range. For 'off' responses the corresponding figures were 10–316 msec for latency-decreasing and 33–177 msec for latency-increasing responses. The maximum shortening (or lengthening) of latency bore no relation to either the threshold latency or the minimum latency, since the slopes of the intensity–latency curves varied greatly for different units.

Response duration and number of spikes per response when plotted against log. intensity also gave two types of linear graph: an increase of duration and spike number with increasing light intensity, and a decrease in duration and spike number with increasing light intensity. Whereas duration and spike number always varied in the same direction for any one unit, the latency in some cases lengthened and in others shortened over the same range of intensity changes.

In some units changes in stimulus intensity produced changes in firing pattern. There were two distinct changes associated with the two basic response types, the bursts and trains.

In 18 units, which at threshold intensity of stimulation responded with a single brief, high-frequency burst of impulses, progressive increase in light intensity caused the appearance of additional bursts. These later bursts appeared sequentially and like the first burst showed shortening of latency with increasing intensity (Fig. 4). Figure 5 shows the mean latencies of individual bursts in multiple burst responses.

In 7 units which at threshold intensity of stimulation responded with a train at either 'on' or 'off', increasing the intensity of the stimulation caused the appearance of a brief pause in the train of spikes. This pause occurred after an initial maximum rate of firing and was followed by a low-frequency discharge. The pause lengthened as the light intensity increased.

Binocular stimulation

In an attempt to find out whether there is any convergence of visual impulses arising in the two eyes on to a single lateral geniculate neurone, we tried the effect of binocular stimulation on 63 single units. Only in 7 of these units was there a response to separate stimulation of the ipsilateral and the contralateral eye. The pattern of the response was not always the same for stimulation of each of the two eyes. Table 1 lists the mean latencies and response patterns of these 7 units. These units were found in areas of transition between regions of response to contralateral and ipsilateral eye stimulation, respectively. Although we have no anatomical evidence it

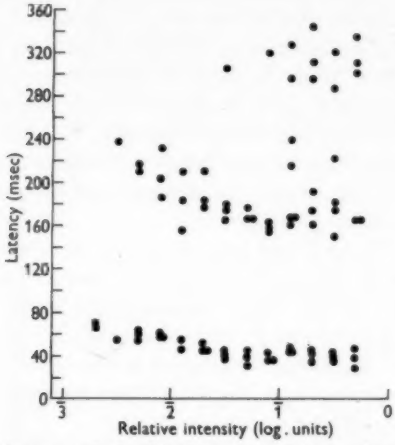

Fig. 4. Intensity–latency relationship for a unit giving multiple burst responses at high intensities of illumination. Each point on the graph indicates a burst response.

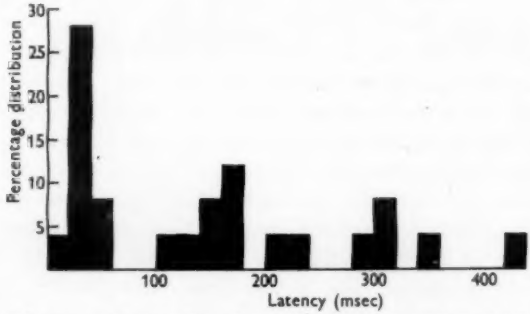

Fig. 5. Histogram of mean latencies of individual bursts in multiple-burst responses. The total number of bursts was 24.

TABLE 1. Latencies (msec) of responses of units activated by separate stimulation of the two eyes

	Ipsilateral eye		Contralateral eye	
Unit	On	Off	On	Off
1 (10)	—	120	—	91
1 (11)	147	89	46	—
3 (11)	96	—	52	44
1 (13)	114	90	—	113
4 (13)	46	65	123	—
5 (13)	230	84	185	73
4 (12)	20	77	—	135

seems likely that these areas of transition correspond to the interlaminar zones.

In a number of units we tried various combinations of ipsilateral and contralateral eye stimulation. This approach revealed the existence of a group of units which responded to stimulation of only one eye, but whose response was modified by the simultaneous stimulation of the other eye. The response of a unit to separate stimulation of the two eyes was easily detected through the loudspeaker and only the 7 units mentioned above showed this type of convergence of impulses from the two eyes. However, to detect modifications in response pattern to stimulation of one eye by simultaneous stimulation of the other eye a detailed quantitative analysis of photographic records was necessary. Of 19 units for which such analyses were done, 13 showed the latter type of binocular interaction. The modifications of response pattern took a number of different forms.

The mean response latencies to stimulation of one eye were increased in 6 units and decreased in 2 units by simultaneous stimulation of the other eye.

In 6 units the rate of firing in response to visual stimulation of one eye was decreased by the illumination of the other eye. This effect occurred when the second eye was illuminated during the response to stimulation of the first. The suppression of the response lasted for the duration of stimulation of the opposite eye; when the illumination of that eye was stopped, the response was resumed at its original rate of firing.

Three units were isolated which responded with multiple bursts at high light intensities. In all cases 'on' responses were recorded to contralateral eye stimulation, but illumination of the ipsilateral eye did not evoke any detectable response. When the ipsilateral eye stimulation was added during the response to contralateral eye stimulation, the subsequent bursts were delayed, and the number of spikes per burst diminished. This effect was more marked, the earlier the stimulation of the ipsilateral eye occurred.

In one unit stimulation of either eye alone produced no response, but stimulation of both eyes together produced an 'on' burst type of response. In one other unit the pattern of the response was altered by stimulation of the two eyes. Ipsilateral eye stimulation gave rise to an 'off' response regularly, but only to an irregular 'on' response; contralateral eye stimulation appeared to be without effect. When the ipsilateral eye was stimulated during the illumination of the contralateral eye, the 'off' response was eliminated, while the 'on' response occurred regularly.

Effects of additional anaesthetic

In order to find out whether some of the very long latencies shown in Figs. 3 and 5 were an effect of the anaesthetic, we studied the effect on

latency of a number of successive doses of sodium pentobarbitone in addition to the initial anaesthetic dose. This was done with four different units. Figure 6 shows the latency changes in one of these units, which gave a double burst response, when a number of doses of sodium pentobarbitone of 9 mg each, were given intravenously. The initial effects of the administration of the anaesthetic were to increase the latencies of

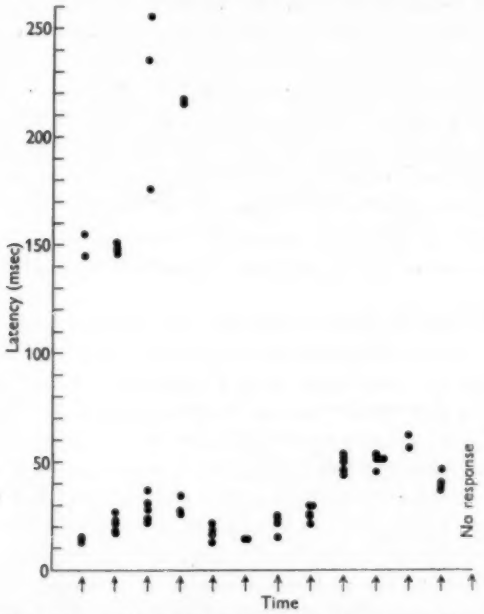

Fig. 6. The effect of increasing depth of anaesthesia on response latency. At each arrow a dose of 9 mg of sodium pentobarbitone was administered intravenously. The unit initially gave a double burst response: after the fourth dose of anaesthetic the second burst disappeared.

both the first and second bursts until the second burst was no longer evoked by the stimulus. Injection of further doses of anaesthetic caused the latency of the first burst to be increased until finally no further response occurred. The maximum increase in latency produced by the additional anaesthetic was 40 msec. For this unit an intensity series was done after the supplementary administration of 27 mg sodium pentobarbitone intravenously. Although all latency values were greater after the extra anaesthetic dose, there was no difference in the slope of the intensity–latency curves.

DISCUSSION

In comparing the responses described in the present study with those recorded from retinal ganglion cells (Kuffler, 1952, 1953) and from single units in the optic tract (Lennox, 1958) the results are best considered under three headings: discharge characteristics, intensity variation and binocular stimulation.

Discharge characteristics

The impulse activity in the absence of illumination recorded here in anaesthetized cats was also found by Hubel (1960) in unanaesthetized animals and is at least partly due to activation from the retina (Bornschein, 1958). Hubel (1960) also described the brief, high-frequency bursts but found that these were abolished by arousal.

The striking variability of responses to a series of identical stimuli may be attributable to both arousal and to the long-lasting excitability changes following activation (Evarts & Hughes, 1957*a*, *b*; Vastola, 1959; Bishop & Davis, 1960).

Intensity variation

Latency-increasing responses were found in the retina (Kuffler, 1952, 1953) but were considered anomalous and due to inhibitory interaction within the receptive fields of light-adapted retinae (Barlow, Fitzhugh & Kuffler, 1957). In the lateral geniculate body 15 % of responses are latency-increasing, and it seems likely that these are due to inhibitory interactions between a number of ganglion cells activated by the diffuse illumination of the retina. Increasing the intensity of illumination is likely to increase the number of active ganglion cells, a proportion of which probably exert an inhibitory effect at the lateral geniculate body. A similar inhibitory mechanism may explain the appearance of pauses in long-sustained responses with an increase in stimulus intensity.

The mechanism of latency-shortening with increasing intensity has been discussed by Rose & Mountcastle (1954) for thalamic units and they suggest that a delay of up to 3 msec can be introduced at one synapse by varying stimulus strength. Even allowing for many more synapses in the pathway investigated in the present study, a change in latency of over 300 msec cannot be explained by lengthening of synaptic delays only, but must involve a change in interneuronal pathway as well. The existence of such polysynaptic pathways is also suggested by the great range of minimum latencies and by the appearance of late bursts in multiple burst responses. Such an explanation for the latter is supported by the finding that the late bursts have a higher threshold and show a greater susceptibility to anaesthetics than the earlier bursts.

Binocular interaction

Although all the older anatomical evidence (Minkowski, 1920; Glees, 1941; Cohn, 1956; Silva, 1956) stressed the complete segregation of fibres from the two eyes in the lateral geniculate body, recently Hayhow (1958) has shown that in the interlaminar zones and in the nucleus interlaminaris medialis there is extensive overlap between fibres from the two eyes. The method of Nauta and Gygax for staining fibres only shows preterminal degeneration and it therefore cannot show whether any one lateral geniculate neurone receives synaptic end feet from fibres coming from the two eyes. Evidence from responses to electrical stimulation of the optic nerves (Bishop *et al.* 1959), and the responses to physiological stimulation described in the present experiments, indicate that this must be so. The number of units fired from both eyes was very small. The units whose response to stimulation of one eye was modified by simultaneous stimulation of the other eye suggest that in these cases the synaptic terminations of fibres from the two eyes may differ qualitatively or quantitatively.

It is interesting to note that most of the modifications which discharge patterns undergo in the lateral geniculate body seem to be inhibitory: this may be the function of the short axon cells which are abundant in the lateral geniculate body of the cat (O'Leary, 1940). It seems then that there is modification in the lateral geniculate body of the retinal discharge pattern coming from each eye as well as some binocular interaction, although the significance of these changes is not understood.

SUMMARY

1. The responses of single units in the lateral geniculate body of the cat to flashes of white light were recorded with extracellular micro-electrodes of tip diameters 2–10 μ. The animals were anaesthetized with sodium pentobarbitone. Most units showed firing in darkness. The responses to light stimulation consisted of a variety of patterns. Successive responses to identical stimuli showed considerable variation.

2. Increase in light intensity above threshold produced changes in all response characteristics: latency in most units was shortened, but in 15% of responses it was lengthened. Response duration and spike number showed similar changes. In some units increase in intensity produced changes in the firing pattern.

3. Latency figures varied widely for different units at a given intensity as well as for a given unit over 4 log. units of intensity change. Administration of supplementary doses of anaesthetic caused an increase in the latency of only 40 msec before no further response could be evoked. The

long latencies recorded in this study do not appear therefore to be due to the anaesthetic effect.

4. Separate stimulation of the two eyes may activate the same unit. However, more often a unit only responded to stimulation of one eye, but its response could be modified by stimulation of the other eye. These modifications involved changes in latency, spike number, response duration, and frequency of firing.

5. The results have been discussed in relation to the occurrence of interaction of impulses at the lateral geniculate body.

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